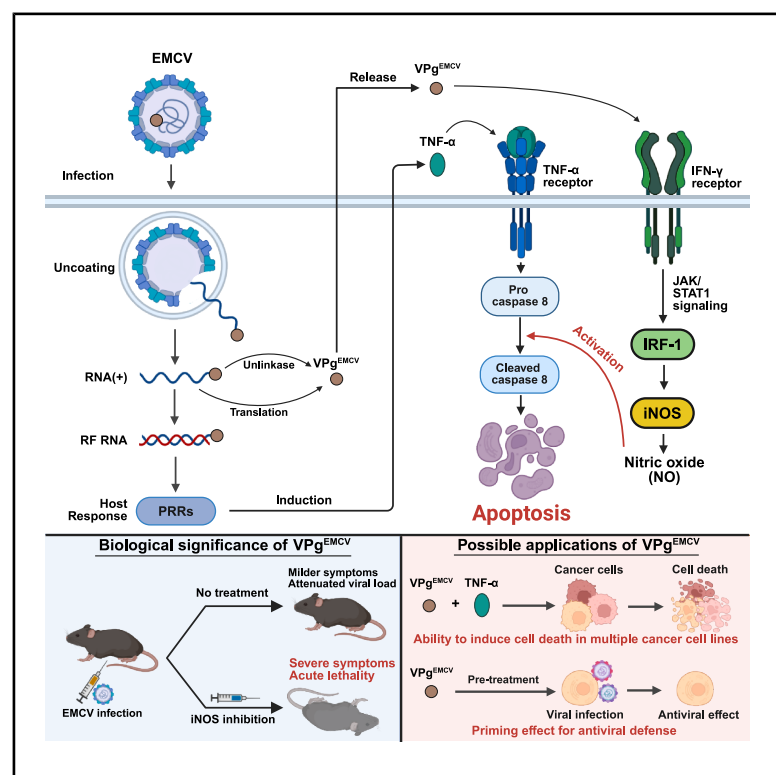


A 20-amino-acid *Cardiovirus* protein exhibits cytokine-mimicry activity to regulate viral replication

Graphical abstract



Authors

Yutaro Shirasaka, Fumihiko Takeuchi, Satoshi Koike, Hiroki Kato, Takashi Fujita

Correspondence

hkato@uni-bonn.de (H.K.),
fujita.takashi.86e@st.kyoto-u.ac.jp (T.F.)

In brief

Shirasaka et al. show that the *Cardiovirus*-derived protein VPg functions as an interferon- γ receptor agonist, activating its canonical signaling cascade that sensitizes cells to TNF- α -triggered cell death. This synergistic cell death reduces virus load, revealing a non-canonical role of VPg in modulating host antiviral responses.

Highlights

- TNF- α and viral protein VPg^{EMCV} are both released from EMCV-infected cells
- VPg^{EMCV} functions as an interferon- γ receptor agonist to activate its signaling cascade
- The synergism of TNF- α and VPg^{EMCV} signaling triggers apoptosis in uninfected cells
- Apoptosis induced by the synergy of TNF- α and VPg^{EMCV} attenuates EMCV load



Article

A 20-amino-acid *Cardiovirus* protein exhibits cytokine-mimicry activity to regulate viral replication

Yutaro Shirasaka,^{1,2} Fumihiko Takeuchi,¹ Satoshi Koike,³ Hiroki Kato,^{4,*} and Takashi Fujita^{1,2,5,*}¹Department of Regeneration Science and Engineering, Institute for Life and Medical Sciences, Kyoto University, Kyoto, Japan²Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kyoto, Japan³Research Center for Infectious Diseases and Medical Sciences, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan⁴Institute of Cardiovascular Immunology, University of Bonn, Bonn, Germany⁵Lead contact*Correspondence: hkato@uni-bonn.de (H.K.), fujita.takashi.86e@st.kyoto-u.ac.jp (T.F.)<https://doi.org/10.1016/j.celrep.2025.116740>

SUMMARY

Viral protein genome-linked (VPg) is a small protein encoded by several non-enveloped positive-sense RNA viruses, including *Cardiovirus*. Although VPg is widely recognized as essential for viral RNA synthesis, its additional roles remain largely unexplored. Here, we report an alternative function of *Cardiovirus* VPg as an interferon- γ receptor agonist. During encephalomyocarditis virus (EMCV) infection, a member of the *Cardiovirus* genus, we uncovered that VPg from EMCV (VPg^{EMCV}) is released extracellularly from host cells. VPg^{EMCV} is then sensed by uninfected host cells, activating the interferon- γ signaling cascade and ultimately producing nitric oxide (NO) through inducible NO synthase induction. NO accumulation is crucial for triggering potent cell death in cooperation with tumor necrosis factor α (TNF- α) signaling, with TNF- α also being induced as an antiviral host response during EMCV infection. We demonstrate that VPg^{EMCV}, comprising merely 20 amino acids, can exhibit cytokine-mimicry activity, with the synergistic interplay between VPg^{EMCV} and TNF- α signaling regulating viral replication and disease pathogenesis.

INTRODUCTION

Viruses must replicate efficiently within the host in the context of host-pathogen interactions, but the viral infection often results in host cell death. Cell death is partly attributable to the viral shutoff of host transcription and/or translation, which are essential for host survival. However, virus-infected cell death largely involves innate immune mechanisms such as caspase activation, suggesting that cell death is induced as a host antiviral response. Thus, death is limited to virus-infected cells and is cell autonomous. Cell death results in the blockade of progeny virus production, which reduces the viral load in the host.¹

The encephalomyocarditis virus (EMCV) is a positive-sense, non-enveloped single-strand RNA virus from the genus *Cardiovirus*.^{2,3} EMCV is known for its lytic nature and its ability to induce cell death in the host.^{4–6} According to several reports, certain EMCV viral proteins alter host apoptosis or other cell-death-related mechanisms, including L and 2A, which inhibit host apoptosis,^{4–6} 2B, which activates the NLR family pyrin domain containing 3 (NLRP3) inflammasome for caspase-1 activation,⁷ and the nonstructural 2C and 3D proteins, which induce autophagy.⁸ Nevertheless, the cell-death molecular mechanisms triggered by EMCV infection and their correlations with its virulence or pathogenicity are not fully understood.

Here, we discovered that cells infected by EMCV produce soluble factors that efficiently trigger cell death in naive uninfected cells in a non-cell-autonomous manner. Surprisingly, the smallest protein (20 amino acids) encoded by EMCV, known as 3B or viral protein genome-linked (VPg), is an essential factor inducing potent cell death. The canonical function of VPg is to serve as a primer for viral RNA synthesis in EMCV.^{3,9} However, no reports have, so far, suggested VPg as having an alternative function to sensitize cells to cell death.

In this study, we discovered that VPg sourced from the genus *Cardiovirus* mimics the action of interferon- γ (IFN- γ), therefore acting as an agonist of the IFN- γ receptor. We aimed to elucidate the molecular mechanism of cell death caused by EMCV and discuss its impact on the viral life cycle and pathogenesis.

RESULTS

EMCV-infected cells produce soluble factors that induce apoptosis in uninfected cells

We infected HeLa cells with the Sendai virus (SeV; *Paramyxoviridae*, *Respirovirus*), the Newcastle disease virus (NDV; *Paramyxoviridae*, *Avulavirus*), the vesicular stomatitis virus (VSV; *Rhabdoviridae*, *Vesiculovirus*), and the EMCV (*Picornaviridae*, *Cardiovirus*) and examined the cell-death mechanisms induced by these viruses (Figure 1A). Here, we used cell staining (Amido



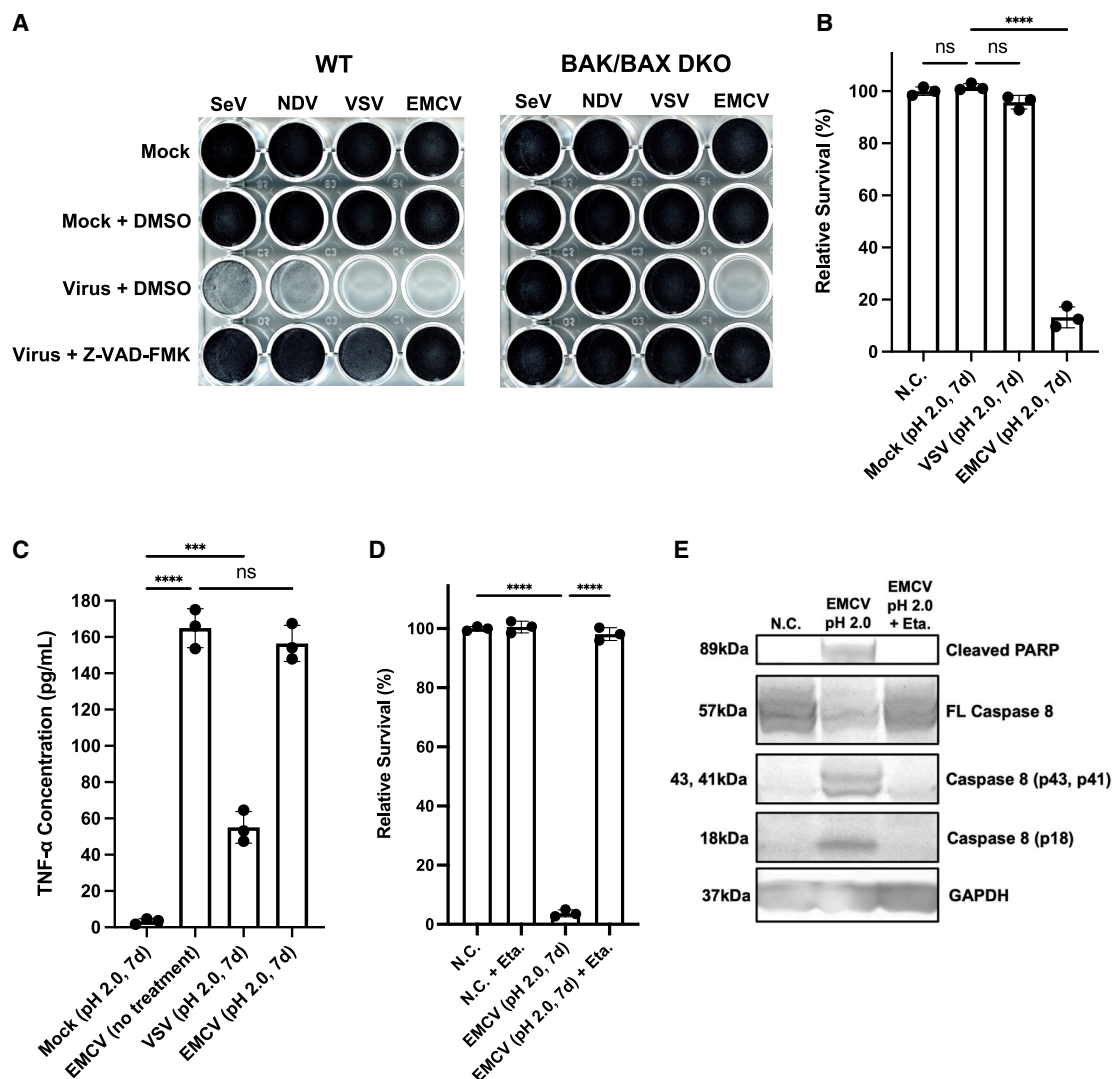


Figure 1. Soluble factors released during EMCV infection induce cell death in uninfected cells

(A) Wild-type and BAK/BAX double-knockout HeLa cells were either mock infected or infected with the indicated viruses (MOI 1.0). The cells were cultivated in a culture medium with DMSO or pan-caspase inhibitor Z-VAD-FMK (10 μ M). Virus infection was performed for 24 h. The cells were fixed and stained with Amido Black 10B.

(B) HeLa cells were mock infected or infected with VSV (MOI 1.0) or EMCV (MOI 1.0) for 24 h. The culture supernatants were collected, acid treated, and neutralized to inactivate viral infectivity (see [STAR Methods](#) for details). Fresh HeLa cells were treated with fresh culture medium (N.C.), acid-treated mock-infected culture medium (Mock [pH 2.0, 7 days]), acid-treated VSV-infected culture medium (VSV [pH 2.0, 7 days]), or acid-treated EMCV-infected culture medium (EMCV [pH 2.0, 7 days]) for 24 h. The cells were fixed and stained with Amido Black 10B, and the relative survival was quantified by elution (see [STAR Methods](#) for details).

(C) HeLa cells were either mock infected or infected with VSV or EMCV (MOI 1.0) for 24 h. The culture supernatants were untreated or acid treated as indicated. The culture supernatants were quantified for TNF- α by ELISA.

(D) Fresh culture medium (N.C.) or acid-treated EMCV-infected culture supernatant was mixed with etanercept (Eta, 10 μ g/mL) for 2 h (controls were maintained under Eta-untreated conditions), then the Eta-TNF- α complex was removed using Protein G beads. Fresh HeLa cells were treated with these mixtures, as indicated, for 24 h. Survival was quantified in the same manner as shown in (B).

(E) HeLa cells were treated with fresh culture medium (N.C.), acid-treated EMCV-infected culture supernatant, or acid-treated EMCV-infected culture supernatant with Eta (depleted in the same manner as in D beforehand) for 9 h. Cell lysates were prepared and subjected to immunoblotting using anti-cleaved PARP, anti-caspase-8, or anti-GAPDH.

In (B)–(D), data are presented as the mean and SD. Statistical significance was determined by one-way ANOVA followed by Tukey's HSD post hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; ns, not significant.

Black 10B) as a proxy readout for cell death. Z-VAD-FMK, a pan-caspase inhibitor, inhibited cell death, demonstrating that these viruses induced programmed death mediated by caspases. In addition, HeLa cells devoid of Bcl-2 homologous antagonist/killer (BAK) and Bcl-2-associated X protein (BAX) generated by gene editing were resistant to cell death by infection with SeV, NDV, or VSV; however, EMCV infection efficiently killed these cells, suggesting that distinct mechanisms mediate EMCV-induced cell death. Use of other fluoromethylketone (FMK) inhibitors revealed that cell death induced by EMCV infection was mainly mediated by caspase-3 and caspase-8 (Figure S1A), suggesting that this cell death closely follows the well-established canonical extrinsic apoptosis pathway.

Based on the evidence of extrinsic apoptosis, we considered the possibility that cell death occurs in a non-cell-autonomous manner and is mediated by secreted factors produced by infected cells. The culture media of virus-infected cells were acidified to lower the pH to 2.0 (treated for 7 days) to inactivate the virus and then neutralized to pH 7.0 to examine the cell-killing activity on fresh cells (Figure 1B). The culture medium of VSV-infected cells did not affect the viability of naive cells. In contrast, the culture medium of EMCV-infected cells robustly induced cell death. This effect was not due to the residual EMCV infectivity (Figure S1B). Acid treatment is known to inactivate viruses while preserving the biological activities of certain cytokines, such as type I IFNs.^{10,11} From the observed cell-death pathway actively involving caspase-8, we considered tumor necrosis factor α (TNF- α) as a potential death-inducing candidate from well-established pathways,¹² so we tested whether TNF- α is detected and active under low pH conditions. Significant TNF- α levels were detected in the culture medium of EMCV- or VSV-infected cells and were preserved post acidification (Figure 1C). I κ B α degradation detected upon treatment with the acid-inactivated EMCV supernatant closely following kinetics of recombinant TNF- α , and its abrogation in TNF receptor 1 (TNFR1)-deficient cells, further support the functional integrity of TNF- α under low-pH conditions (Figure S1C).

To examine the possible involvement of TNF- α in this cell death, the acid-treated culture supernatant of EMCV-infected cells was treated with etanercept (Eta), a dimeric fusion protein composed of the soluble TNF receptor and the Fc portion of immunoglobulin G1 for neutralizing TNF- α . Eta was removed from the supernatant beforehand using Protein G beads. Cell-killing activity was completely abrogated after treatment with this supernatant (Figure 1D), and poly[ADP-ribose] polymerase (PARP) and caspase-8 cleavage were also blocked by Eta (Figure 1E). These results were also replicated using TNFR1-deficient HeLa cells (Figures S1D and S1E). Quantification of cellular ATP further validated the cytotoxicity of the supernatant in wild-type (WT) cells, while no cytotoxicity was observed after TNF- α depletion by Eta or in TNFR1-deficient cells (Figure S1F). These results indicated that EMCV infection induced death-inducing soluble factor production, wherein TNF- α appeared to be an essential component.

TNF- α and a <3 kDa low-molecular-weight factor are synergistic drivers of apoptosis during EMCV infection

We hypothesized that other factors may contribute to efficient cell death in addition to TNF- α , considering that the use of

10 ng/mL TNF- α alone did not induce cell death. To investigate this, the mock-infected and EMCV-infected cell-culture supernatants were subjected to ultrafiltration using membranes with molecular weight cutoffs of 50 kDa, 10 kDa, and 3 kDa (Figure 2A). The 50 kDa and 10 kDa membranes successfully retained viral particles (Figure S2A) and TNF- α (Figure S2B), respectively. Notably, even at one-tenth of its original concentration, the <50 kDa filtrate alone induced cell death (Figure 2B), replicating the results obtained with acid inactivation shown in Figure 1. The <50 kDa >10 kDa fraction, which contained most proteins (Figure S2C) including TNF- α (Figure S2B), did not induce potent cell death on its own (Figure 2B). The <10 kDa and <3 kDa filtrates also did not induce cell death on their own. However, the addition of recombinant TNF- α to only the <10 kDa or <3 kDa fractions restored cell-death-inducing activity, suggesting the presence of a factor in the <3 kDa fraction acting synergistically with TNF- α (Figure 2C). Quantification of DNA fragmentation showed the same trend, further confirming that potent cell death is triggered when TNF- α is added to the <10 kDa or <3 kDa fractions (Figure S2D). Although we cannot completely rule out the contribution of some additional cytokines or proteins in the culture supernatant, these results support our hypothesis that the EMCV-infected culture supernatant contains at least two key components that act synergistically to induce cell death: TNF- α and a low-molecular-weight factor smaller than 3 kDa.

This factor was resistant to acid (pH 2.0, 7 days) and heat (100°C, 10 min; Figure 2D). So far, this factor was specific to EMCV infection, so we tested other members of *Picornaviridae*, including poliovirus (PV), coxsackievirus B3 (CVB3), and enterovirus 71 (EV71). The culture supernatants of HeLa cells infected with these viruses were filtered through a 10K membrane and subjected to cell-death assays (Figure 2E). Cell death was not detectable other than with EMCV infection, even in the presence of TNF- α (10 ng/mL). Accordingly, we hypothesized that the phenomenon was strongly associated with *Cardiovirus* infection.

Identification of VPg^{EMCV} as a low-molecular-weight apoptosis-sensitizing factor derived from EMCV

Since the observed cell death was restricted to EMCV, we hypothesized that an EMCV-derived factor may correspond to the low-molecular-weight factor. As the size of this factor was suggested as <3 kDa, one candidate was the smallest protein encoded by EMCV, known as 3B or VPg, which consisted of 20 amino acids (2.3 kDa). Figure 3A displays the comparison of the VPg sequences of cardioviruses and enteroviruses. The eight C-terminal amino acids of *Cardiovirus* VPg are highly conserved; however, those of *Enterovirus* are much less conserved.

To test this hypothesis, we obtained an EMCV VPg peptide via chemical synthesis (VPg^{EMCV}) and used it in a cell-death assay (Figure 3B). The cells treated with a high concentration of VPg^{EMCV} (1 μ g/mL and 100 ng/mL) alone induced strong cell death. At intermediate concentrations (10 ng/mL and 1 ng/mL), VPg^{EMCV} alone did not induce cell death but rather efficiently killed the cells in the presence of TNF- α . Because of the sequence homology, we assessed the VPg activity of the Saffold virus (VPg^{Saffold}), Theiler's murine encephalomyelitis virus (VPg^{TMEV}), and poliovirus (VPg^{PV}). As shown in Figure 3C,

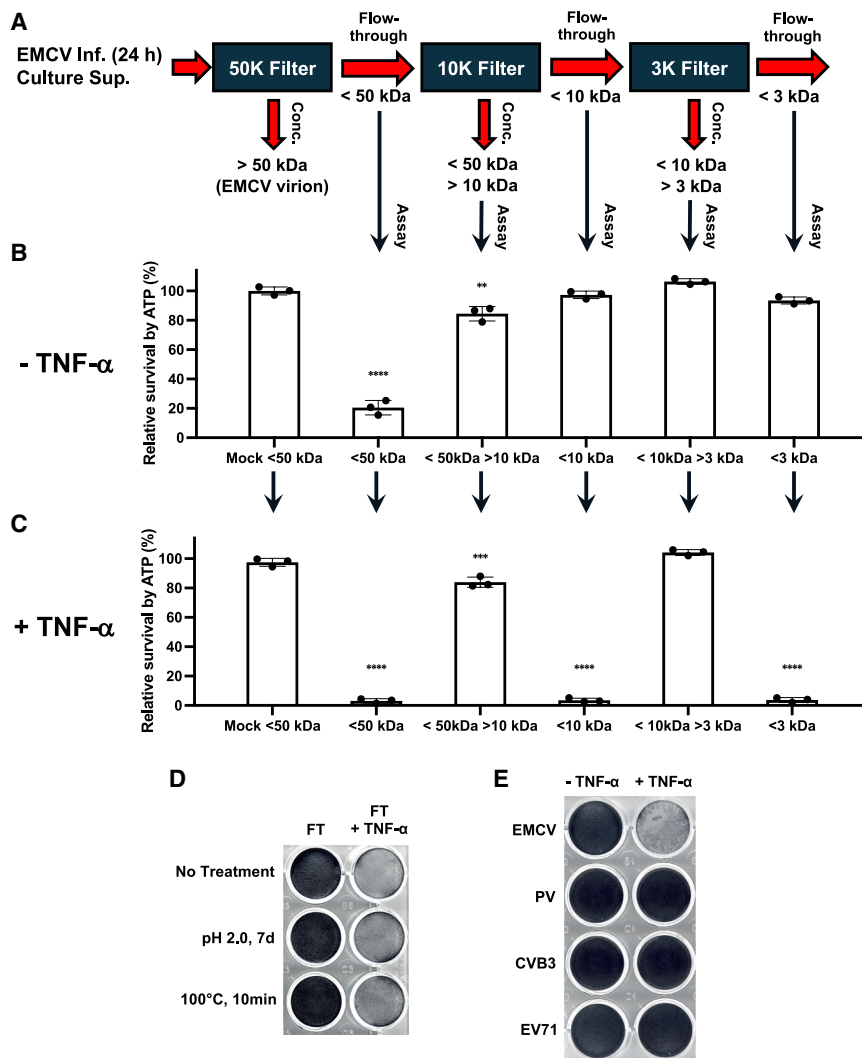


Figure 2. A low-molecular-weight compound is released during EMCV infection to sensitize host cells to cell death, specific to cardioviruses

(A–C) (A) Schematic of the experiment. HeLa cells were mock infected or infected with EMCV (MOI 0.1) for 24 h. Mock and EMCV culture supernatants were fractionated with Amicon Ultra Filtration membranes. Amicon Ultra 50K flowthrough from EMCV (<50 kDa) was further filtered using Amicon Ultra 10K. The flowthrough (<10 kDa) and the concentrated supernatant (>10 kDa) were collected. The flowthrough of Amicon Ultra 10K was further filtered using Amicon Ultra 3K. The concentrated supernatants (>10 kDa, >3 kDa) were adjusted to their original volumes. All the collected fractions were then diluted 10-fold using DMEM (10% FBS), treated to HeLa cells, (B) without TNF-α or (C) with TNF-α (10 ng/mL) for 24 h. Relative survival for (B) and (C) were determined by ATP quantification (CellTiter-Glo).

(D) HeLa cells were infected with EMCV (24 h, MOI 0.1), and the culture supernatant was filtered by Ultra 10K. The filtrate was untreated, acid treated (pH 2.0, 7 days), or heat treated (100°C, 10 min). These samples were analyzed for cell-killing activity using fresh HeLa cells without or with TNF-α (10 ng/mL). The cells were then fixed and stained in the same manner as shown in Figure 1A.

(E) HeLa cells were infected with EMCV, PV, CVB3, and EV71 at MOI 1.0. The culture supernatants were collected 24 h post infection and filtered using Amicon Ultra 10K. The flowthrough was collected and treated with fresh HeLa cells without or with TNF-α (10 ng/mL). The cells were fixed and stained in the same manner as shown in Figure 1A.

In (B) and (C), data are presented as the mean and SD. Statistical significance was determined with one-way ANOVA followed by Tukey's HSD post hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

VPg^{EMCV}, VPg^{Safford}, and VPg^{TMEV} but not VPg^{PV} exhibited strong cell-death induction in cooperation with TNF-α. At a high concentration (100 ng/mL), VPg^{PV} exhibited efficient cell death only in the presence of TNF-α, suggesting that VPg^{PV} has cell-death-inducing properties similar to those of VPg^{EMCV}, but its activity was around one-hundredth that of VPg^{EMCV} or other *Cardiovirus* VPgs (Figure S3A).

To explore this phenomenon further, we focused on the importance of the eight C-terminal amino acids conserved in *Cardiovirus*. To test this aspect, we synthesized chimeric VPg, consisting of 14 N-terminal amino acids of VPg^{PV} and eight C-terminal amino acids of VPg^{EMCV} (VPg^{P-EMCV}) (Figure 3D). VPg^{P-EMCV} exhibited a more than 100-fold increase in the death-inducing activity compared to VPg^{PV}, suggesting an important role of the eight C-terminal amino acids in efficient cell killing. To further elucidate the structure-function relationship, we tested the N-terminal deletion mutants of VPg^{EMCV} (Figure S3B). VPg^{EMCV} with only 17 amino acids (VPg^{EMCV17AA}) exhibited a slightly reduced activity (50%) compared to full-length VPg^{EMCV}; how-

ever, the deletion to 13 amino acids (VPg^{EMCV13AA}) or eight amino acids (VPg^{EMCV8AA}) resulted in its complete inactivation. These results suggested that, in addition to the highly conserved eight amino acids, less-conserved residues of VPg^{EMCV} participate in the cell-killing activity. Since the third tyrosine is the site for covalent linkage with viral genomic RNA,¹³ we tested a mutant in which tyrosine was replaced with phenylalanine (VPg^{EMCV-F}). This tyrosine mutation did not alter its cell-death-inducing activity (Figure 3D), implying that the cell-death activity of VPg^{EMCV} is independent of its canonical RNA-linking function.

To identify the exact residues critical for the function of VPg^{EMCV}, we generated alanine mutants for each of its 20 amino acid residues, except for the seventh residue, which is already alanine. HeLa cells were then treated with each single-residue VPg^{EMCV} mutant in combination with recombinant TNF-α (Figure 3E). Mutations at the 15th glutamine (Q) and 16th leucine (L) residues resulted in markedly impaired activity. We next compared the activities of WT VPg^{EMCV} with the Q15A mutant, the L16A mutant, and the double mutant (Q15A/L16A). When

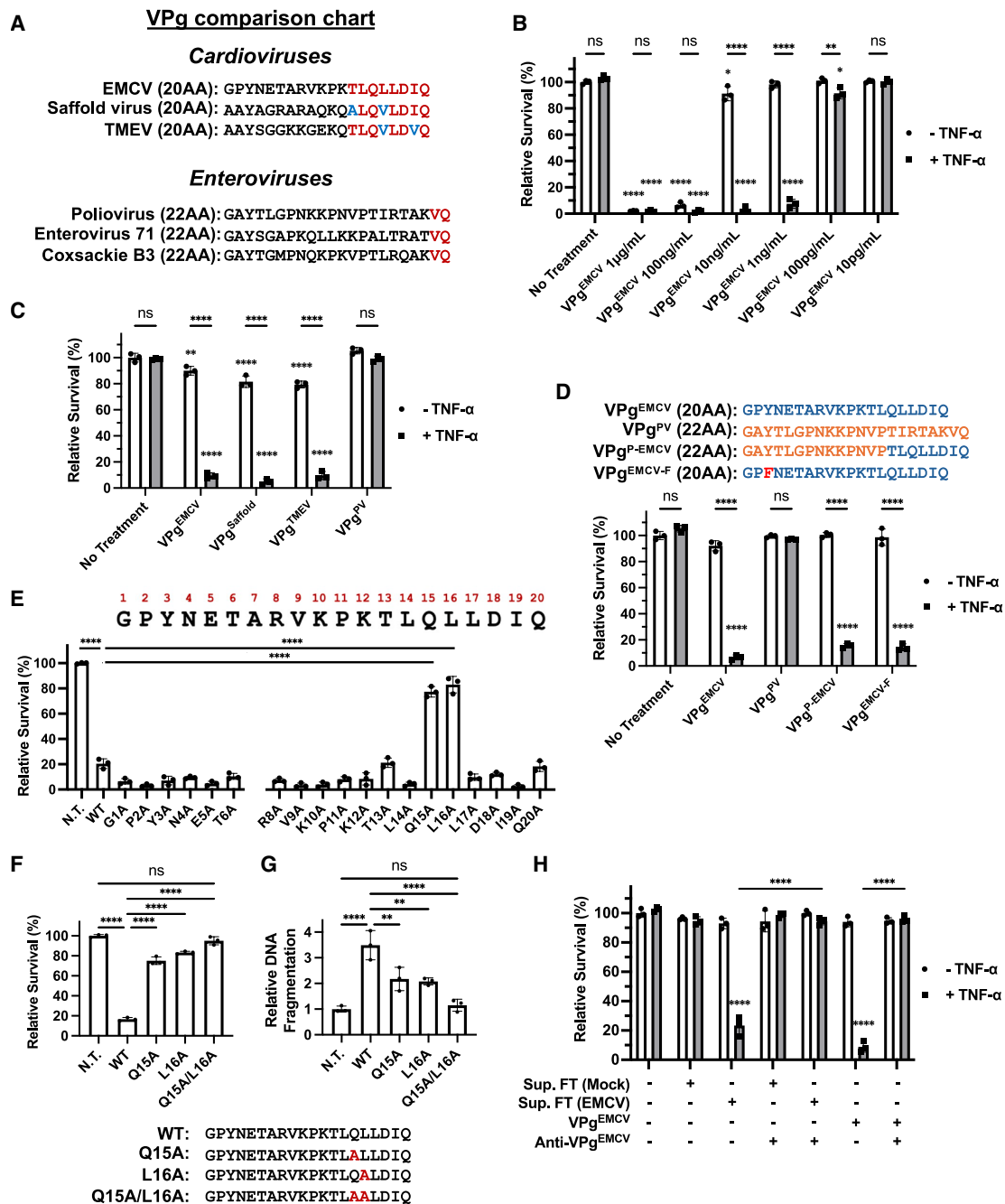


Figure 3. Identification of VPg^{EMCV} and critical C-terminal amino acid residues for triggering TNF-α-synergistic apoptosis

(A) Comparison of different VPgs of cardioviruses and enteroviruses. Conserved sequences of the C terminus are shown in red, and the changes to closely related amino acids are shown in blue.

(B) HeLa cells were treated with synthetic VPg^{EMCV} at the indicated concentrations for 24 h, without or with TNF-α (10 ng/mL). The cells were fixed and stained, and the relative survival was quantified as shown in Figure 1B.

(C) HeLa cells were treated with synthetic VPg^{EMCV}, VPg^{Saffold}, VPg^{TMEV}, or VPg^{PV} at 1 ng/mL for 24 h, without or with TNF-α (10 ng/mL). The cells were fixed and stained, and the relative survival was quantified as shown in Figure 1B.

(D) HeLa cells were treated with the synthetic VPgs as shown in the chart at 1 ng/mL for 24 h, without or with TNF-α (10 ng/mL). The EMCV sequences are shown in blue, those of PV are in orange, and the tyrosine-to-phenylalanine mutation is shown in red. The cells were fixed and stained, and the relative survival is quantified as shown in Figure 1B.

(E) A series of Ala substitutions at the indicated positions of VPg^{EMCV} (top) were generated. HeLa cells were treated with these mutants (10 ng/mL) in the presence of recombinant TNF-α (10 ng/mL) for 24 h. The cells were fixed and stained, and the relative survival was quantified as shown in Figure 1B.

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treated together with TNF- α , the double mutant exhibited an even greater reduction in cell-death-inducing activity (Figure 3F) and DNA fragmentation (Figure 3G) compared to the single mutants. These findings indicate that the C-terminal residues, particularly both Q15 and L16, are critical for the pro-apoptotic function of VPg^{EMCV}.

To validate that VPg^{EMCV} was the death factor in the EMCV-infected culture medium, we raised a polyclonal antibody against VPg^{EMCV} (see STAR Methods). The cell-death-inducing activity of the EMCV-infected culture medium, as well as that of synthetic VPg^{EMCV}, was efficiently eliminated by adsorption using the anti-VPg^{EMCV} antibody (Figure 3H). This antibody detected VPg^{EMCV} by immunoblotting in EMCV-infected cells and the post-infected culture supernatants (Figure S3C), albeit with slower gel mobility compared to the reference synthetic VPg^{EMCV}.

Given that natural VPg exists as conjugate with -pU or -pUpU,¹⁴ suggesting that the differences in gel mobility and band patterns between natural and synthetic VPg^{EMCV} are due to VPg-pU modifications, we performed digestion of natural VPg^{EMCV} using snake venom phosphodiesterase I (PDEI), as described previously.¹⁵ PDEI digestion of natural VPg^{EMCV} resulted in the appearance of a smear similar to the synthetic VPg^{EMCV} (Figure S3D), confirming that the gel mobility difference is due to -pU modifications.

Because of the difference of electrophoretic band patterns, we attempted to quantify the concentration of VPg^{EMCV} using dot blotting using our antibody. Using the reference synthetic VPg^{EMCV}, we estimated the concentration of VPg^{EMCV} in the EMCV-infected cell-culture supernatant to be approximately 17 ng/mL (Figure S4A). We then compared the activity of natural and synthetic VPg^{EMCV} by adjusting their concentrations accordingly. Serial dilutions of both natural and synthetic VPg^{EMCV}, starting from the same concentration (1 ng/mL), resulted in very similar cell-death-inducing activities together with TNF- α (Figure S4B), suggesting no distinct differences in activity between the two forms. VPg^{EMCV} was also successfully detected in the <50 kDa, <10 kDa, and <3 kDa filtrates from Figure 2 (Figure S4C). Notably, the kinetics of EMCV virion production nearly paralleled the death-inducing activity (the release of sufficient VPg^{EMCV} in the supernatant to induce cell death) in the culture medium (Figure S4D). These results strongly suggest that this death-inducing low-molecular-weight compound in the EMCV-infected culture supernatant is VPg^{EMCV} and that its -pU modification does not alter its activity.

We then determined the VPg^{EMCV} and TNF- α concentrations to induce efficient cell death (Figure S4E). TNF- α at 1 ng/mL ap-

peared as the saturating concentration for rapid cell killing together with VPg^{EMCV}, and 0.1 ng/mL TNF- α was found to be sufficient to induce cell death, also following close tendencies observed in Figure 2. Considering that EMCV infection induced approximately 0.15–0.3 ng/mL TNF- α (Figures 1C and S2B), these results strongly suggest that TNF- α and VPg^{EMCV} are exclusive components that induce programmed cell death in the culture supernatant of EMCV-infected cells.

Several human and mouse cells were tested for cell-death induction by VPg^{EMCV}, TNF- α , or their combination (Figure S4F). All the tested cells were resistant to cell-death induction by single treatment with VPg^{EMCV} or TNF- α , except L929, which exhibited partial cell death by TNF- α . Dramatic synergism of VPg^{EMCV} and TNF- α was observed in all tested cells, showing that this cell-death phenomenon is common to numerous cell types.

VPg^{EMCV} mimics IFN- γ signaling to induce iNOS/NO-dependent cell death and antiviral responses

Several reports have previously described that TNF- α and IFN- γ synergistically trigger cell death.^{16–18} This cell-death pathway includes the induction of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production. To test whether EMCV-induced cell death involves NO production, we examined the effect of NOS inhibitors (Figure S5A). Both iNOS-specific and universal NOS inhibitors efficiently blocked EMCV-induced cell death 24 h post infection. In addition, these inhibitors blocked cell death by VPg^{EMCV} and TNF- α (Figure S5B). These results imply that VPg^{EMCV} mimics IFN- γ in the synergism with TNF- α for efficient cell death. To validate this point, p-STAT1 and iNOS induction were examined (Figure 4A). The cells treated with TNF- α alone exhibited a basal level of iNOS and p-STAT1 (Y701) and undetectable cleavage of caspase-8. However, treatment with IFN- γ or VPg^{EMCV} alone dramatically induced both iNOS and p-STAT1. The combination of TNF- α and IFN- γ or VPg^{EMCV} induced caspase-8 cleavage, which is consistent with previous reports and our present observations. STAT1 phosphorylation at tyrosine 701 by JAK1 is an immediate-early response after IFN- γ association with its cognate receptor chains. iNOS induction and STAT1 phosphorylation by either IFN- γ or VPg^{EMCV} was efficiently blocked by tofacitinib, a JAK1 inhibitor (Figure S5C), suggesting that VPg^{EMCV} signaling is very similar to IFN- γ signaling from the initial steps of the pathway.

To monitor NO induction, we quantified the nitrite levels in cells treated with IFN- γ and VPg^{EMCV} (Figure S5D). SIN-1, an NO donor, was used as a positive control that could efficiently produce nitrite. As expected, IFN- γ treatment induced significant nitrite levels. Similar nitrite levels were detected in cells treated

(F) HeLa cells were treated with VPg^{EMCV} (WT), VPg^{EMCV-Q15A} (Q15A), VPg^{EMCV-L16A} (L16A), or VPg^{EMCV-Q15A/L16A} (Q15A/L16A) (10 ng/mL) in the presence of recombinant TNF- α (10 ng/mL) for 24 h. The cells were fixed and stained, and the relative survival was quantified as shown in Figure 1B.

(G) HeLa cells were treated with the same synthetic VPgs as in (F) for 9 h. Cells were washed using PBS, lysed, centrifuged, and the supernatant collected for DNA fragmentation quantification using ELISA. DNA fragmentation values are calculated relative to the mean values of the negative control (N.T.) as 1.

(H) HeLa cells were either mock infected or infected with EMCV (MOI 0.1) for 24 h. The culture supernatant was collected and heated (100°C, 10 min) to inactivate the virus. The culture supernatant and synthetic VPg^{EMCV} solution (2 ng/mL in DMEM, 10% FBS) were immunoprecipitated (using Dynabeads Protein G, without or with anti-VPg^{EMCV} antibody binding) overnight. The VPg^{EMCV}-depleted samples were diluted 2-fold with fresh DMEM (10% FBS) and subjected to a HeLa cell-death assay using the same protocol as shown in Figure 1B.

In (B)–(H), data are presented as the mean and SD. Statistical significance was determined by one-way ANOVA (E–G) or two-way ANOVA (B–D, H) followed by Tukey's HSD post hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; ns, not significant.

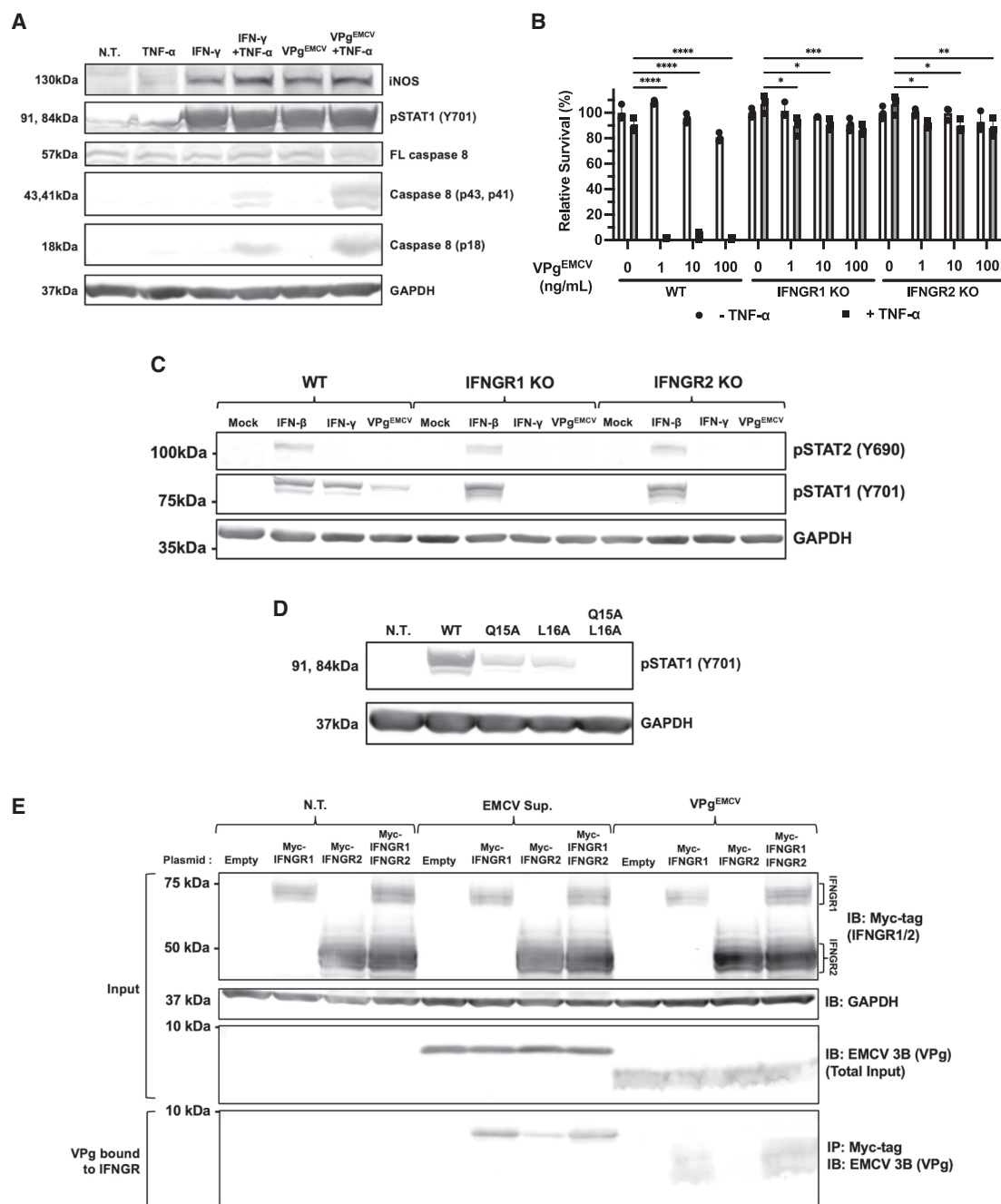


Figure 4. VPg^{EMCV} functions as an IFNGR1/2 agonist by binding to IFNGR and exhibiting IFN- γ -like activity

(A) HeLa cells were treated with TNF- α (10 ng/mL), IFN- γ (10 ng/mL), or VPg^{EMCV} (1 ng/mL) as shown for 9 h, and the cell lysates were collected for immunoblotting using anti-iNOS, anti-pSTAT1 (Y701), anti-caspase-8, and anti-GAPDH.

(B) Wild-type (WT) HeLa, IFNGR1 KO HeLa, and IFNGR2 KO HeLa cells were treated with VPg^{EMCV} at the indicated concentrations without or with TNF- α (10 ng/mL). The cells were fixed and stained, and their relative survival was quantified as shown in Figure 1B. Data are presented as the mean and SD. Statistical significance was determined by two-way ANOVA followed by Tukey's HSD post hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

(C) WT, IFNGR1 KO, and IFNGR2 KO HeLa cells were treated with either IFN- β (1000 U/mL), IFN- γ (10 ng/mL), or VPg^{EMCV} (1 ng/mL) for 9 h, and lysates were subjected to immunoblotting using anti-pSTAT2 (Y690), anti-pSTAT1 (Y701), and anti-GAPDH.

(D) HeLa cells were treated with 10 ng/mL VPg^{EMCV} (WT), VPg^{EMCV-Q15A} (Q15A), VPg^{EMCV-L16A} (L16A), or VPg^{EMCV-Q15A/L16A} (Q15A/L16A) for 1 h, and lysates were subjected to immunoblotting using anti-pSTAT1 (Y701) and anti-GAPDH.

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with VPg^{EMCV}, VPg^{Saffold}, and VPg^{TMEV}. However, nitrite induction by VPg^{PV} was significantly lower than that by VPg^{EMCV} and almost the same as that of the control cells. Tofacitinib efficiently inhibited nitrite induction by VPg^{EMCV} (Figure S5E), and cell death induced by VPg^{EMCV} and TNF- α was also inhibited (Figure S5F). iNOS induction by IFN- γ signaling also includes the previous induction step of transcription factor IRF-1.^{16,19} We tested the effects of silencing of IRF-1 and iNOS (Figure S5G), which rescued the cell-death event induced by VPg^{EMCV} and TNF- α .

IFN- γ initiates signaling by interacting with the ectodomain of IFN- γ receptor complexes (a hetero-tetramer of IFNGR1 and IFNGR2) and activating JAK1 kinase associated with the receptor's cytoplasmic domain. To elucidate the involvement of IFNGR1 and IFNGR2 in VPg^{EMCV} signaling, we generated knockout (KO) HeLa cells by gene editing (Figure S5H). IFNGR1 and IFNGR2 KO cells were then assessed for VPg^{EMCV}-induced cell death (Figure 4B). WT cells were highly sensitive to VPg^{EMCV} (1 ng/mL) in the presence of TNF- α (10 ng/mL). However, IFNGR1 or IFNGR2 KO cells did not display cell death at as high as 100 ng/mL VPg^{EMCV} under the same conditions, suggesting that a functional IFN- γ receptor is essential for VPg^{EMCV} signaling. Loss of STAT1 phosphorylation was also confirmed in these KO cells after IFN- γ or VPg^{EMCV} treatment, but not after IFN- β treatment, which utilizes IFNAR1 and IFNAR2 (Figure 4C). Notably, STAT2 phosphorylation (Y690) was exclusively detected in IFN- β -treated cells but not in those treated with IFN- γ or VPg^{EMCV}, showing more similarities between IFN- γ and VPg^{EMCV}. A time-course comparison of STAT1 phosphorylation revealed very similar kinetics between IFN- γ and VPg^{EMCV} (Figure S6A), further indicating that both initiate signaling via identical receptor complexes and mechanisms, which also contradicts the idea that this signaling could result from autocrine IFN- γ .

We also tested the ability of the Q15A, L16A, and Q15A/L16A mutant EMCV VPgs, which showed loss of cell-death-inducing activity in Figures 3F and 3G, to induce STAT1 phosphorylation (Figure 4D). The Q15A and L16A mutants exhibited markedly weaker p-STAT1 bands compared to WT VPg^{EMCV} at 10 ng/mL, while the Q15A/L16A double mutant showed no STAT1 phosphorylation activity. These results indicate that the Q15 and L16 residues are critical for initiating IFNGR-JAK/STAT1 signaling, and that simultaneous substitution at both sites completely abolishes the ability to phosphorylate STAT1.

Having confirmed that IFNGR1/2 is the receptor for which VPg^{EMCV} acts as an agonist, we next examined whether VPg^{EMCV} physically interacts with the IFNGR complex. To test this, we transfected HEK293T cells with Myc-tagged human IFNGR1 and IFNGR2 expression vectors and performed a pull-down assay for VPg^{EMCV} using an anti-Myc tag antibody. Before lysate collection, cells were treated with either filtered EMCV culture supernatant or synthetic VPg^{EMCV} for 1 h. Cells expressing either Myc-IFNGR1 or Myc-IFNGR2, but not those of control,

co-precipitated natural and synthetic VPg^{EMCV} (Figure 4E). Interestingly, the immunoprecipitation bands were stronger in IFNGR1-expressing than IFNGR2-expressing cells. These results confirm that VPg^{EMCV} physically interacts with IFNGR1/2 for signal transduction.

We further compared binding between VPg^{EMCV} and the VPg^{EMCV-Q15A} mutant in a similar co-immunoprecipitation assay. While WT VPg^{EMCV} was associated with IFNGR1/2, VPg^{EMCV-Q15A} did not co-precipitate with the receptors (Figure S6B), coinciding with attenuation of its biological activity (Figures 3E–3G) as well as attenuated STAT1 activation (Figure 4D). We note that we were unable to confirm the loss of binding for the L16A or Q15A/L16A mutants, as our anti-VPg^{EMCV} antibody could not efficiently detect these mutant peptides.

Because IFN- γ is a well-known cytokine with antiviral activity, we examined the antiviral activity of VPg^{EMCV}. VPg was subjected to a conventional antiviral bioassay based on cell protection from VSV-induced cell death (see STAR Methods). Similar to its cell-death-inducing activity (Figure 3C), VPg^{EMCV}, VPg^{Saffold}, and VPg^{TMEV} exhibited clear antiviral activity comparable to that of IFN- γ (Figure S6C). Consistent with its lower cell-killing activity, VPg^{PV} also exhibited lower antiviral activity. A VSV yield reduction assay also confirmed the antiviral effect of the VPgs (Figure S6D), lowering the VSV titer to below the detection limit ($<1 \times 10^3$ plaque-forming units) for VPg^{EMCV} and VPg^{EMCV-F}.

VPg^{EMCV} upregulates genes in common with IFN- γ and downregulates distinct genes

To elucidate the target genes of VPg^{EMCV} and IFN- γ signaling, transcripts of cells treated with IFN- β , IFN- γ , and VPg^{EMCV} were analyzed by deep sequencing. IFN- β was tested as a control cytokine that induces p-STAT2 and p-STAT1. Conversely, IFN- γ and VPg^{EMCV} exclusively induce p-STAT1 (Figure S5C). The results shown by volcano plots suggested that these stimuli upregulated several genes, which is consistent with those induced by IFNs, termed IFN-stimulated genes (ISGs) (Figure 5A). Genes upregulated by VPg^{EMCV} were compared with those induced by IFN- β and IFN- γ . Genes upregulated by IFN- β and VPg^{EMCV} (the top 20 and 21–50) are listed in Figure 5B. Several known ISGs were commonly upregulated by IFN- β and VPg^{EMCV} (six common genes in the top 20 and five common genes in the top 21–50). However, a comparison between IFN- γ and VPg^{EMCV} revealed that more genes were commonly upregulated (14 common genes in the top 20 and 18 common genes in the top 21–50) (Figure 5C).

Among the top 50 VPg^{EMCV}-induced genes, 25 were selectively and highly upregulated only by IFN- γ and VPg^{EMCV} (Figure S7A). Furthermore, since IFNs regulate human leukocyte antigen (HLA) genes and play critical roles in acquired immune responses, we assessed their expression. As shown in Figure S7B, class I HLAs such as HLA-A, HLA-B, and HLA-C were upregulated by IFN- β , IFN- γ , and VPg^{EMCV}. In contrast,

(E) HEK293T cells were mock transfected (only Lipofectamine 2000) or with expression vectors for Myc-IFNGR1 (6 μ g), Myc-IFNGR2 (6 μ g), or their combination (total 12 μ g) for 48 h. The cells were then treated with either N.T. (no treatment), 10 $\times$ concentrated EMCV-infected supernatant (HeLa, 24 h post infection, MOI 1.0, <50 kDa filtered), or 100 ng/mL synthetic VPg^{EMCV} for 1 h, then lysates were prepared in 200 μ L of lysis buffer. 100 μ L of each lysate was pulled down using Protein G-anti-Myc tag conjugate and eluted using 40 μ L of elution buffer. 20 μ L (1/2) of eluate, and 1/5 of lysates (input), as well as 1/5 of total added VPg being subjected to immunoblotting using anti-Myc tag, anti-VPg^{EMCV}, and anti-GAPDH.

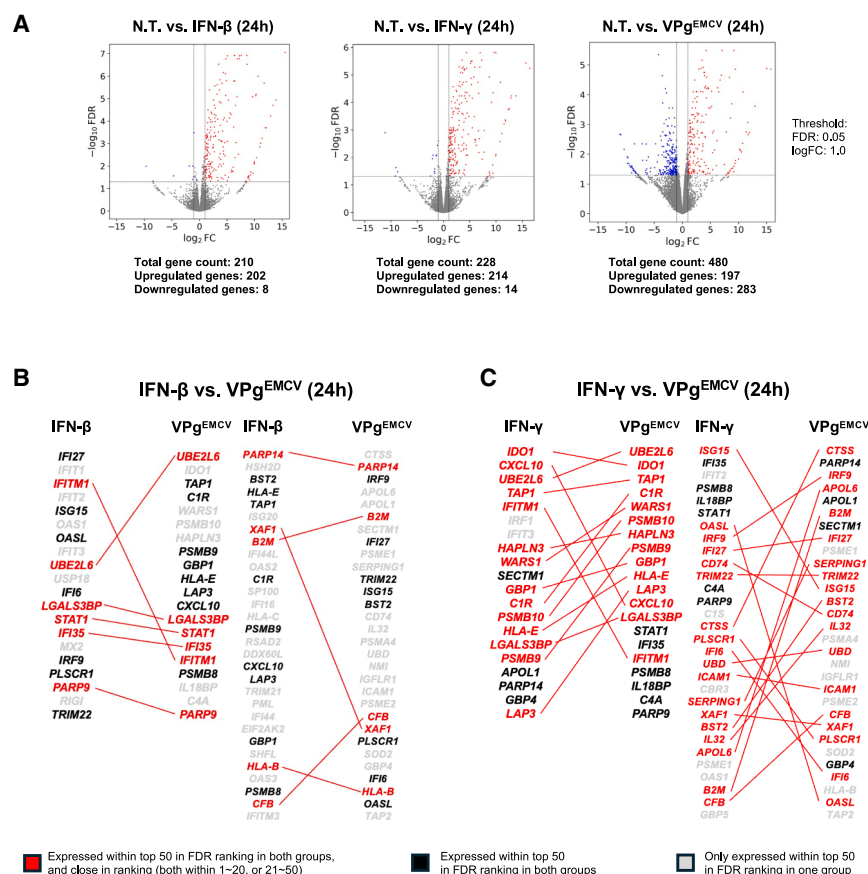


Figure 5. Comparison of the target genes regulated by IFN- β , IFN- γ , and VPg^{EMCV}

HeLa cells were treated with either IFN- β (1,000 U/mL), IFN- γ (10 ng/mL), or VPg^{EMCV} (1 ng/mL) for 24 h (including non-treated [N.T.]). RNA was extracted, and RNA-seq analysis was performed ($n = 2$ per group).

(A) Volcano plots showing upregulated (red dots) or downregulated (blue dots) genes by IFN- β , IFN- γ , and VPg^{EMCV} compared to N.T. Threshold values (values considered differentially expressed) are false discovery rate (FDR) = 0.05, log(fold change) = 1.0.

(B) Gene upregulation comparison induced by IFN- β and VPg^{EMCV}. Comparison of top 1–20 (left) and top 21–50 (right) upregulated genes by FDR ranking (comparing N.T. vs. IFN- β and N.T. vs. VPg^{EMCV}) are shown. The red genes are those induced by both IFN- β and VPg^{EMCV} and close in ranking. The genes in black are the top 50 of that induced by both IFN- β and VPg^{EMCV}, but not in close ranking (definition: one is in the top 1–20 and the other is in the top 21–50). The gray genes are those induced by either IFN- β or VPg^{EMCV} but not induced by both stimuli.

(C) Genes upregulated by IFN- γ and VPg^{EMCV} are compared as in (B).

Finally, to determine whether VPg^{EMCV}-mediated gene downregulation is dependent on IFNGR, WT and IFNGR1/2 double-knockout (DKO) HeLa cells were treated with IFN- β , IFN- γ , or

class II HLA including HLA-DRA, HLA-DMB, and HLA-DPB1 were selectively upregulated by IFN- γ and VPg^{EMCV} (Figure S7C). Among the upregulated genes identified by RNA sequencing (RNA-seq), the top two genes, UBE2L6 and IDO1, were further validated by real-time PCR. The results confirmed that these genes were selectively upregulated by IFN- γ and VPg^{EMCV} in time-dependent (Figure S7D) and dose-dependent (Figure S7E) manners.

Despite gene upregulation, IFN- β and IFN- γ downregulated eight and 14 genes, respectively; however, VPg^{EMCV} downregulated 283 genes (Figure 5A). A heatmap of the top 50 (Figure S8A) downregulated genes by VPg^{EMCV} indicated that most of these genes were not downregulated by IFN- γ or IFN- β . Notably, several known proto-oncogenes, including DUSP1, MYC, IER2, TCIM, CHAC1, and ZBED3, were selectively downregulated by VPg^{EMCV} (Figure S8B). The top two downregulated genes, H4C3 and DUSP1, were validated by real-time PCR and shown to be reduced in both time-dependent (Figure S8C) and dose-dependent (Figure S8D) manners. While not as pronounced as with VPg^{EMCV}, H4C3 was slightly downregulated by IFN treatments, particularly by IFN- β . DUSP1, on the other hand, was downregulated only by VPg^{EMCV}. It is also notable that downregulation of H4C3 and DUSP1 by VPg^{EMCV} occurred early (1–3 h post treatment, Figure S8C), whereas upregulation of UBE2L6 and IDO1 occurred at later time points (6–12 h post treatment, Figure S7D).

VPg^{EMCV}. The top five upregulated and downregulated genes by VPg^{EMCV} were then compared between WT and IFNGR1/2 DKO cells using real-time PCR. First, upregulation of the top five ISGs by both IFN- γ and VPg^{EMCV} was completely abolished in IFNGR1/2 DKO cells (Figures S9A and S9B). Unexpectedly, however, downregulation of the top five genes by VPg^{EMCV} was still observed in both WT and IFNGR1/2 DKO cells (Figure S9C). Notably, a comparison between WT and DKO cells revealed that gene downregulation was more pronounced in WT cells (all <5% compared to no treatment), whereas IFNGR1/2 DKO cells retained 10%–40% expression relative to no treatment. These results suggest that gene downregulation by VPg is possibly regulated by both IFNGR-dependent and -independent mechanisms; however, these details require further investigation.

VPg^{EMCV}-mediated cell death limits EMCV replication and attenuates lethality in vivo

In this study, we discovered that, during EMCV infection, VPg^{EMCV} is released from infected cells and cooperates with the cytokine TNF- α to induce iNOS-mediated programmed cell death. The significance of iNOS during EMCV replication was examined to explore the biological impact of this phenomenon. WT HeLa cells, HeLa IFNGR1/2 DKO, and HeLa CASP8 KO were infected with EMCV, and viral titers were determined after 24 h (Figure 6A). The viral yield in HeLa IFNGR1/2 DKO and HeLa

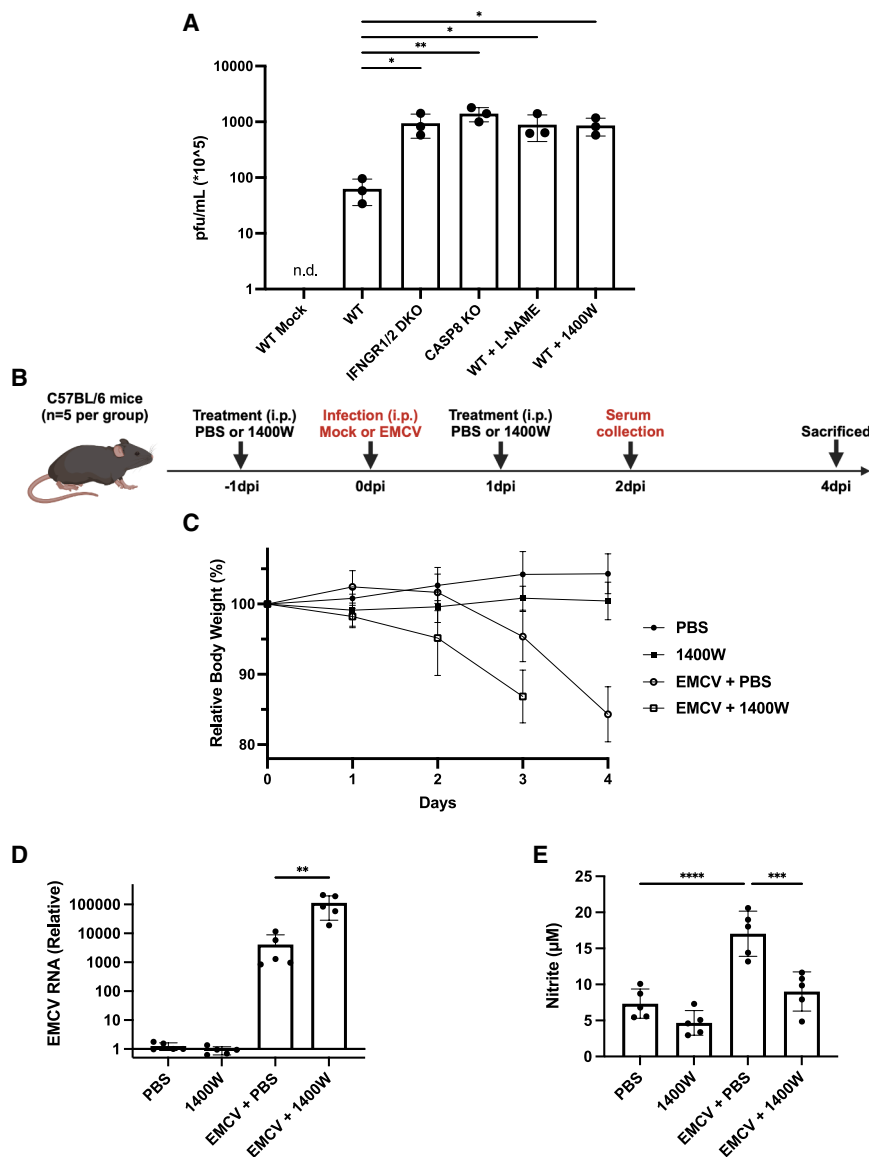


Figure 6. Biological significance of the IFN- γ -like activity of VPg^{EMCV} during EMCV infection

(A) Wild-type (WT) HeLa, as well as IFNGR1/IFNGR2 DKO HeLa and CASP8 KO HeLa, were mock infected or infected with EMCV (MOI 0.1) for 24 h. WT HeLa cells were pre-treated for NOS inhibition with L-NAME (1 mM) or 1400W (100 μ M) 2 h before infection and were similarly infected with EMCV for 24 h in the presence of inhibitors. The viral yield was determined by plaque assay. n.d., not detected (below 1×10^5 PFU/mL).

(B) Schematic of the mouse (C57BL/6 strain, $n = 5$) infection by EMCV.

(C) Mouse body weights were measured daily and plotted.

(D) Relative EMCV RNA levels of the serum at day 2 post infection, as determined by RT-qPCR.

(E) Nitrite levels of the serum at day 2 post infection were quantified by Griess quantification.

In (A), (C), (D), and (E), data are presented as the mean and SD. In (A), (D), and (E), statistical significance was determined by one-way ANOVA followed by Tukey's HSD post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

their sacrifice at day 4. EMCV-infected mice showed reduced body weight on days 3 and 4. The 1400W treatment of EMCV-infected mice rapidly reduced their body weight on days 2 and 3. Body weight differences of 1400W-treated or non-treated mice for days 2 and 3 are highlighted in Figure S10A. Daily symptoms of the individual mice are summarized in Table S1, which highlights individual symptom progression for each mouse. While mice without iNOS inhibition remained relatively healthy on day 3 post infection, severe paralysis was observed as early as day 3 in 1400W-treated mice. All 1400W-treated mice died by day 4 post infection, whereas all

CASP8 KO were significantly enhanced. EMCV infection in the presence of NOS inhibitors also enhanced the viral yield. These results suggest that the blockade of IFNGR1/2, iNOS, or caspase-8, essential for VPg^{EMCV}-induced cell death, results in an enhanced EMCV yield. Given that deletion of IFNGR1/2, iNOS, and caspase-8 all lead to similarly increased viral loads, we interpret that the abrogation of cell death directly correlates with increased EMCV titers and that iNOS induction, through subsequent NO accumulation, specifically contributes to caspase-8-dependent cell death.

Next, the effect of the iNOS inhibitor, 1400W, was examined in a mouse infection model. The mice (C57BL/6 strain) were mock infected or infected with EMCV intraperitoneally (day 0) and administered with PBS or 1400W, as indicated in Figure 6B. Their body weight was monitored daily (Figure 6C). Uninfected mice treated with PBS or 1400W maintained their body weight until

mice without iNOS inhibition, although weakened and with some paralyzed, remained alive. This observation of rapid mouse death following intraperitoneal EMCV infection (approximately 3–6 days) is consistent with data from previous reports.²⁰

The body weight loss and symptoms suggest that inhibiting iNOS, which is directly linked to VPg^{EMCV}-induced cell death, increased EMCV virulence. The quantification of EMCV RNA in the serum on day 2, which we interpret as a proxy for viral load in the blood, showed that 1400W treatment significantly increased viral replication (Figure 6D). EMCV infection also increased nitrite levels in the serum at day 2. This effect was significantly decreased by 1400W treatment (Figure 6E). Consistent with these results, *Nos2* and *Tnf* expressions were downregulated by 1400W administration (Figures S10B and S10C). These results implied that iNOS/NO-induced cell death during

EMCV infection decreases the viral yield *in vitro* and *in vivo* and represses host lethality *in vivo*.

DISCUSSION

We found that cells infected with EMCV produce factors that efficiently induce programmed cell death in uninfected cells. These factors comprise the host protein TNF- α and the viral protein VPg^{EMCV}. Figure 7 summarizes the signaling cascade of cell death induced by TNF- α and VPg^{EMCV}. EMCV infection induces viral genomic RNA release in the cytoplasm (step 1). The genomic RNA serves as mRNA and is translated into polypeptide, which undergoes proteolytic processing into functional proteins. VPg^{EMCV} is covalently modified at the third amino acid, tyrosine, with pU or pUpU and functions as a primer for viral RNA synthesis.^{3,9} As a result, VPg^{EMCV} gets covalently attached at the 5' end of the genomic RNA. VPg^{EMCV} is excised by the host enzyme tyrosyl-DNA phosphodiesterase 2 (TDP2), also known as VPg unlinkase.^{9,21} The defects in TDP2 cause growth retardation of enteroviruses,^{14,21} suggesting that VPg uncoupling is critical for efficient viral replication.

We discovered that extracellular VPg^{EMCV} functions as a cytokine mimic and demonstrated that natural and synthetic VPg^{EMCV} exhibit very similar activities (Figure S4B), suggesting that both VPg-pU and free VPg may function similarly in this context. We also confirmed that the differences in band patterns between natural and synthetic VPg (Figures S3C and S3D) are due to VPg-pU modifications.

Whether extracellular VPg^{EMCV} mainly originates from polypeptide processing or uncoupling from viral RNA remains to be elucidated. It is certain, however, that VPg^{EMCV} can be detected in the culture supernatant of EMCV-infected cells (Figure S3C). We have observed that release of VPg into the supernatant occurs with kinetics that closely mirror EMCV virion release (Figure S4D). Given that picornaviruses like EMCV are non-enveloped, cell lysis is the predominant release mechanism. It is well established that viral infection can alter cell-membrane permeability and induce cytosolic leakage. Because the cytoplasmic marker GAPDH is simultaneously detected in the culture supernatant, we currently believe that extracellular detection of VPg is likely a consequence of virus-induced cytopathic effects.

Host-pathogen recognition receptors then sense viral double-stranded RNA (dsRNA) generated during replication to trigger antiviral signals that induce cytokines, including TNF- α (step 2). The secreted TNF- α binds to TNFR1 and triggers pro-apoptotic signaling in the cytoplasm (step 3). Extracellular VPg^{EMCV} interacts with IFNGR1/2 to trigger signals similar to those induced by IFN- γ in the cytoplasm: STAT1 phosphorylation, IRF1 and iNOS induction, and NO production (step 4). NO accumulation sensitizes cells to cell death, synergistically cooperating with pro-apoptotic TNF- α signaling to execute caspase activation and cell death, as in mechanisms previously reported.^{16,17}

Our results showed that despite having no structural similarity with IFN- γ , VPg^{EMCV} mimics IFN- γ to activate IFNGR1/2 (Figure 4B) and the target genes (Figure 5). Furthermore, we detected physical associations of VPg^{EMCV}, IFNGR1, and IFNGR2 (Figures 4E and S6B), supporting a hypothesis that VPg^{EMCV} reinforces the receptor-ligand complex to transduce signals.

The action of human and mouse IFN- γ is species specific; human IFN- γ has a >100-fold stronger effect on human cells than mouse cells, and vice versa.²² Interestingly, our results showed that VPg^{EMCV} exhibits similar biological activity in both human and mouse cells (Figure 3F). VPg^{EMCV} and IFN- γ upregulate common genes, including several ISGs and HLA molecules (Figures 5 and S7). Conversely, VPg^{EMCV} downregulates far more genes than IFN- γ . Genes downregulated by VPg^{EMCV} (Figures S8A and S8B) include several proto-oncogenes, such as the well-known MYC. Another gene, DUSP1, negatively regulates the MAP kinase cascade to repress cell death,²³ therefore becoming a potential target for cancer therapy. These results suggest a possible contribution of these genes to cell death induced by VPg^{EMCV}. VPg^{EMCV} regulates target genes common to IFN- γ and a set of unique genes. Structural studies have shown that the IFN- γ dimer binds to the hetero-tetramer IFNGR1/2 to form a hetero-hexamers, thereby triggering cytoplasmic signals.²⁴ In the context of VPg^{EMCV}, although we cannot completely rule out the possibility that VPg^{EMCV} forms a dimer similar to IFN- γ , this appears unlikely based on the current data, as active VPg^{EMCV} was recovered from the <3 kDa filtrate (Figure S4C). Previous mutational studies revealed that modified IFN- γ binds to non-canonical IFNGR1/2 or IFNGR1/1 dimers and transmits signals, resulting in the development of distinct patterns of target gene expression.^{24–26}

From these past reports, we initially hypothesized that VPg^{EMCV} forms a complex with IFNGR1/2 distinct from the IFN- γ /IFNGR1/2 complex, leading to activation of JAK1 and JAK2 and resulting in the induction of both common and unique target genes. This idea was also based on previous reports showing that IFNGR1/2 mediates distinct biological effects depending on the bound IFN- α subtype.^{27,28} However, our data show that cells lacking IFNGR1/2 still exhibited downregulation of these unique genes (Figure S9C), while ISG upregulation was completely abolished (Figures S9A and S9B). These results raise a possibility that VPg^{EMCV} possesses a yet unknown function independent of IFNGR1/2. Nevertheless, our data also indicate that gene downregulation is partly dependent on IFNGR1/2, as the extent of downregulation was more pronounced in WT cells compared to IFNGR1/2-deficient cells (Figure S9C). Although this unexplained phenomenon remains to be investigated, we propose that while VPg^{EMCV} may have functions not yet reported, its ability to downregulate specific genes likely occurs synergistically with IFNGR signaling.

The IFN- γ -like activity of VPg^{EMCV} is primarily specified by the eight C-terminal amino acids, and we further confirmed that the Q15 and L16 residues are crucial for VPg^{EMCV} activity (Figures 3E–3G and 4D) and IFNGR binding (Figure 4E). This suggests that direct binding to IFNGR should involve these two amino acids in the C-terminal region. Moreover, as immunoprecipitation analysis revealed much stronger binding of VPg to IFNGR1 than to IFNGR2 (Figure 4E), we speculate that Q15 and L16 may contribute to a strong interaction with IFNGR1. However, the eight C-terminal amino acids alone are biologically inactive, and removing three N-terminal amino acids can significantly impair the VPg activity, indicating that all 20 residues are required for VPg to be a complete ligand for IFNGR1/2. VPg^{PV} exhibited low activity; however, grafting the eight C-terminal

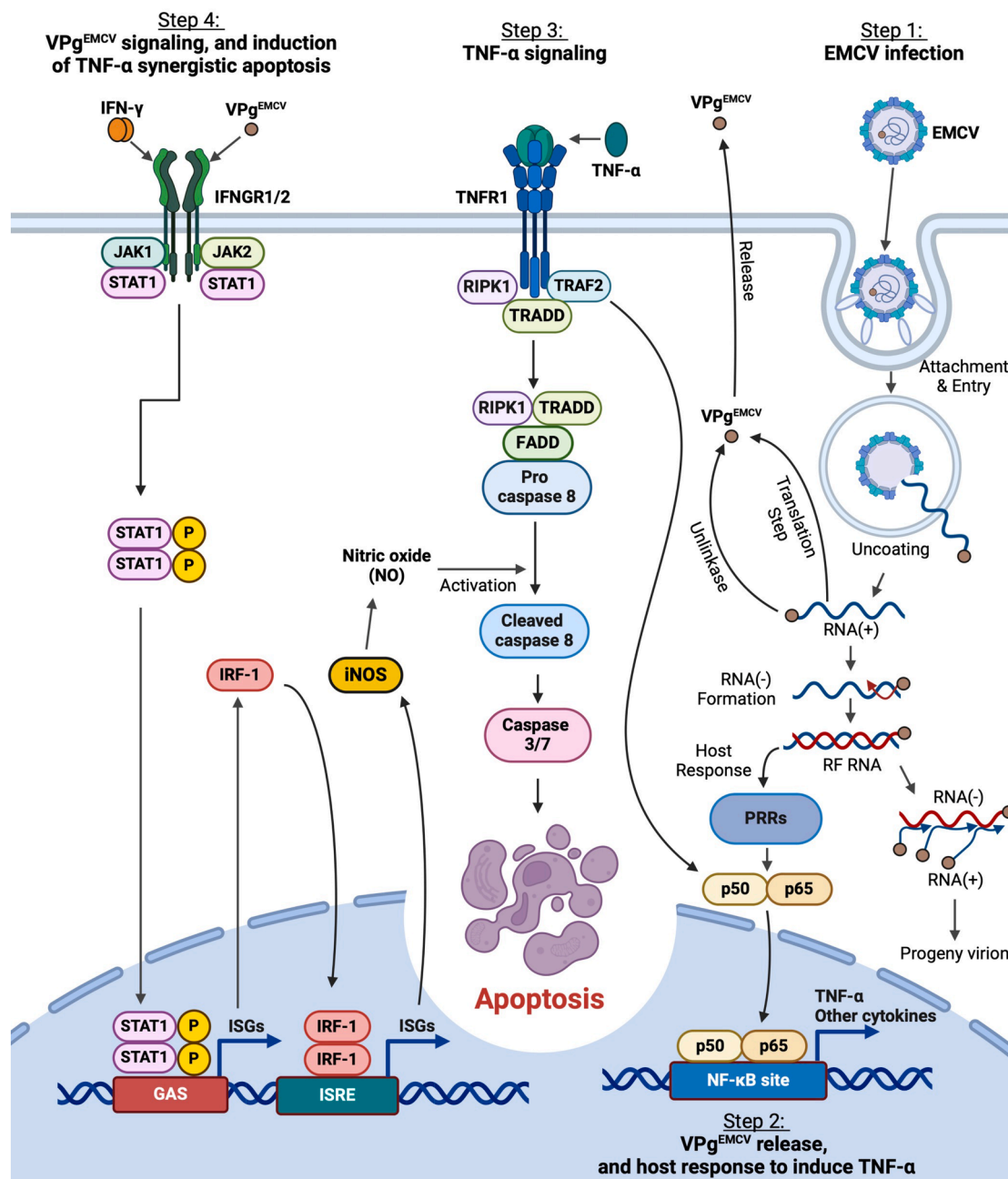


Figure 7. Schematic representation of the signaling pathways displaying synergism between TNF-α and VPg^{EMCV} signaling

During EMCV infection, EMCV enters the cell, and viral RNA is released into the cytoplasm. VPg^{EMCV}, which serves as a primer during replication, is detached by VPg unlinkase, and VPg^{EMCV} is also generated during translation. VPg^{EMCV} is then released extracellularly (step 1). The replicative form of viral RNA (dsRNA) is sensed by host-pathogen recognition receptors (PRRs), which trigger antiviral signals to induce inflammatory cytokines, including TNF-α (step 2). TNF-α binds to TNFR1, which sends its downstream signals (step 3). VPg^{EMCV} mimics IFN-γ signaling through IFNGR1/2, and NO is generated through the JAK/STAT1-IRF1-iNOS-signaling pathway. The synergism of TNF-α signaling and VPg^{EMCV} signaling ultimately induces cell apoptosis after caspase cleavage (step 4).

amino acids of VPg^{EMCV} into VPg^{PV} conferred full biological activity. Moreover, VPg^{TMEV} and VPg^{Saffold}, both of which have highly conserved C termini compared to VPg^{EMCV} but less conserved N termini, continued to exhibit cell-death-sensitizing activity similar to that of VPg^{EMCV}. These results suggest that the 12 N-terminal amino acids of the *Cardiovirus* VPgs and 14

N-terminal amino acids of the poliovirus VPg retain common biological structures despite no clear primary sequence conservation. Taken together, we speculate that although direct binding with IFNGR occurs particularly at residues Q15 and L16, the overall length of the N-terminal region, rather than its specific sequence, is also critical for efficient receptor interaction. It is

tempting to speculate that VPgs such as VPg^{PV} are a prototype and the *Cardiovirus* incorporates eight C-terminal residues, particularly the conserved 15th glutamine and 16th leucine (or valine), which are absent in *Enterovirus* VPgs, to fully acquire IFN- γ -like activity.

We speculate that the IFN- γ -like activity of VPg^{EMCV} could have at least two significant impacts. First, blocking the cell-death pathway by gene deletion or drug administration increases the viral yield *in vitro*. In addition, in the mouse infection model, iNOS inhibition increased the serum viral levels, which coincided with the development of severe symptoms and early mouse death. This could be interpreted simply as inhibiting cell death, which could prolong virion production. It has been reported that IFN- γ or TNF- α administration in mice resulted in therapeutic effects against EMCV infection.^{29–31} Based on our findings, this therapeutic effect could be a consequence of decreased viral production through enhanced cell death in addition to the intrinsic antiviral activity of IFN- γ . The acquisition of IFN- γ -like activity in VPg^{EMCV} may have resulted in partial viral attenuation to allow the survival of the host, thereby enhancing viral transmission to a new host.

The second possible role of VPg^{EMCV} is related to viral pathogenesis in tissues, although this may not be directly related to the EMCV life cycle. A study claims that TMEV infection causes encephalitis, particularly hippocampal cell death; however, viral replication has been detected in the non-hippocampal portion of the brain, and cell death appears to happen in a non-cell-autonomous manner.³² Apoptotic markers and signs of damage by reactive oxygen species in the hippocampus were detected, as mentioned in this cited study, resembling the apoptosis we observed. VPg^{EMCV} may contribute to tissue damage by cell killing, although the dying cells are not infected or resistant to infection. Therefore, cell death by VPg^{EMCV} and TNF- α may function as a double-edged sword by reducing viral load and enhancing tissue pathogenesis. In this regard, the iNOS inhibitor in our mouse infection model may not reach the brain to exert its protective effects due to the blood-brain barrier. Thus, the local blockade of VPg^{EMCV}-induced cell death may be beneficial in counteracting lethal tissue damage.

This study revealed that VPg^{EMCV} mimics IFN- γ , a central innate and adaptive immunity coordinator. Owing to its simple structure, VPg^{EMCV} is easy to synthesize in large quantities and with high purity, and it is amenable to multiple variant designs. Therefore, VPg^{EMCV} could be a candidate therapeutic drug for antiviral treatment and immunomodulation, including the induction of HLA and natural killer cells. Furthermore, the drastic apoptosis induced by VPg^{EMCV} and TNF- α suggests their promising potential in targeting malignant tumors. Our findings in this report provide a new aspect of virus-protein-induced host cell death in virulence and pathogenicity, and we have also provided possibilities for utilizing VPg^{EMCV} for clinical applications.

Limitations of the study

To better elucidate the biological impact of the IFN- γ agonist-like function of VPg, further research using recombinant EMCV or other picornaviruses with engineered VPg is deemed crucial. However, the feasibility of this experiment is questionable, as mutating VPg will most likely disrupt viral replication. It will be

difficult to determine whether the observed results are due to altered replication or disruption of the IFN γ agonist-like activity of VPg, making experiments with mutant VPgs highly challenging. Furthermore, more *in vitro* and *in vivo* studies are needed to strengthen the possibility of using VPg^{EMCV} as a future drug candidate. Evaluating the efficacy and toxicity of VPg^{EMCV}, especially *in vivo*, would also be essential. The precise extracellular release mechanism of VPg is another important question to address, particularly if we are to further dissect and discuss the unexplored roles of VPg in the EMCV life cycle. Finally, although we have presented direct evidence regarding VPg-IFN γ 1/2 binding by immunoprecipitation and identified crucial residues required for signaling initiation, this study remains limited by the lack of supporting evidence to generate a complete model for VPg-IFN γ binding. Particularly, how the Q15 and L16 residues bind to IFN γ (especially to IFN γ 1, which shows stronger binding), why the N-terminal length is necessary for efficient binding and activity, the binding affinity, and the binding status of VPg (including whether dimerization occurs) remain to be determined. This, and the release mechanism of VPg, are both areas substantial enough to be the focus of future studies. Also, as discussed earlier, the peculiar observation that certain genes are still downregulated in the absence of IFN γ , albeit less drastically than in WT cells, highlights another limitation that requires further investigation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests should be directed to and will be fulfilled by the lead contact, Takashi Fujita (fujita.takashi.86e@st.kyoto-u.ac.jp).

Materials availability

Unique reagents generated in this study are available from the [lead contact](#) upon reasonable request.

Data and code availability

- RNA-seq data have been deposited in the Gene Expression Omnibus and are publicly available as of the date of publication (accession number GEO: GSE286568).
- This paper does not report original code.
- Additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon reasonable request.

ACKNOWLEDGMENTS

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TRR 237 (369799452 to H.K. [B22]). Figures 6B and 7, and the graphical abstract, were created using [BioRender.com](https://www.biorender.com).

AUTHOR CONTRIBUTIONS

Y.S., H.K., and T.F. conceptualized the study and designed the methodology; Y.S., F.T., S.K., and T.F. performed all the experiments in this study; Y.S. generated the HeLa KO cell lines used in this study, compiled the data of RNA-seq analysis, created the figures, and performed statistical analyses; F.T. and Y.S. designed and performed the mouse infection experiments; S.K. provided the *Enterovirus* strains used for this study and performed the infection experiments with PV, CBV3, and EV71; H.K. assisted in study design and provided critical input for improving the study; Y.S. and T.F. wrote the manuscript; all authors reviewed the manuscript; T.F., H.K., and Y.S. acquired the funding for this study; and T.F. provided overall supervision for this project.

DECLARATION OF INTERESTS

We have filed a patent (pending, serial #2025-062342) for the IFN- γ receptor agonist-like function of VPgs described in this study and for its potential clinical applications (listing T.F., Y.S., F.T., and H.K. as inventors, and Kyoto University as applicant).

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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 - Transfection
 - CRISPR-Cas9 knockout cell line generation
 - Amido Black 10B cell survival assay
 - Quantitative cell survival analysis
 - Cell survival analysis by ATP quantification
 - Bradford assay
 - SDS-PAGE and immunoblotting
 - Virus infection
 - Viral inactivation methods
 - Plaque assay
 - Enzyme-linked immunosorbent assay (ELISA)
 - Neutralization of TNF- α
 - RNA extraction
 - RT-qPCR
 - Size-dependent separation of compounds
 - Griess quantification
 - Synthetic peptides
 - Antiviral activity assays
 - Mice infection model
 - Confirmation of VPg^{EMCV} by immunoblotting
 - Phosphodiesterase I (PDEI) digestion of VPg^{EMCV} and immunoblotting
 - Quantification of VPg^{EMCV} in the EMCV-infected supernatant
 - Concentration adjustment of natural and synthetic VPg^{EMCV}
 - Anti-VPg^{EMCV} antibody generation
 - VPg^{EMCV} depletion using pulldown
 - Co-immunoprecipitation (binding assay) of IFNGR1/2 and VPg^{EMCV}
 - RNA-seq and bioinformatic analyses
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-GAPDH	Santa Cruz Biotechnology	Cat#: sc-32233; RRID: AB_627679
Anti-caspase-8	Cell Signaling Technology	Cat#: 9746; RRID:AB_2275120
Anti-Cleaved PARP (Asp214)	Cell Signaling Technology	Cat#: 9541; RRID:AB_331426
Anti-3B (Anti-VPg ^{EMCV})	This paper (Created by Immuno-Biological Laboratories Co., Ltd.)	N/A (see Method Details)
Anti-Mengo 3DPol	Santa Cruz Biotechnology	Cat#: sc-65633; RRID:AB_1565377
Anti-iNOS	Santa Cruz Biotechnology	Cat#: sc-7271; RRID:AB_627810
Anti-STAT1	Cell Signaling Technology	Cat#: 9172; RRID:AB_2198300
Anti-pSTAT1 (Y701)	Cell Signaling Technology	Cat#: 9167; RRID:AB_561284
Anti-pSTAT2 (Y690)	Cell Signaling Technology	Cat#: 4441; RRID: AB_2198445
Anti-Myc-Tag	Cell Signaling Technology	Cat#: 2276; RRID: AB_331783
Anti-IkBα	Santa Cruz Biotechnology	Cat#: sc-371; RRID: AB_2235952
Anti-HSP70	Santa Cruz Biotechnology	Cat#: sc-32239; RRID: AB_627759
Anti-IFNGR1	Proteintech	Cat#: 10808-1-AP; RRID: AB_2121604
Anti-IFNGR2	Proteintech	Cat#: 10266-1-AP; RRID: AB_2121709
Anti-mouse IgG, AP-linked (secondary antibody)	Cell Signaling Technology	Cat#: 7056; RRID:AB_330921
Anti-rabbit IgG, AP-linked (secondary antibody)	Cell Signaling Technology	Cat#: 7054; RRID: AB_2099235
Bacterial and virus strains		
Encephalomyocarditis virus (EMCV)	ATCC	Cat#: VR-129B
Sendai Virus (SeV), strain Cantell	ATCC	Cat#: VR-907
Newcastle Disease Virus (NDV), strain Miyadera/51	Fujita et al., 1979 ³³	N/A
Vesicular Stomatitis Virus (VSV), strain Indiana	Oh et al., 2016 ³⁴	N/A
Poliovirus (PV), strain type I Mahoney	Shiroki et al., 1995 ³⁵	N/A
Coxsackie Virus B3 (CVB3), strain Nancy	Klump et al., 1990 ³⁶	N/A
Enterovirus 71 (EV71), strain Isehara VP1-145Q variant	Kobayashi et al., 2018 ³⁷	N/A
Competent high DH5α	TOYOBO	Cat#: DNA-903F
Biological samples		
Human Dermal Fibroblasts, adult (HDFa)	PromoCell	Cat#: C-12302
Embryonated Chicken Eggs (9-day old)	SHIMIZU Laboratory Supplies Co., Ltd.	N/A
Chemicals, peptides, and recombinant proteins		
Dulbecco's Modified Eagle Medium (DMEM)	Nacalai Tesque Inc.	Cat#: 16971-55
Fetal bovine serum (FBS)	Sigma-Aldrich	Cat#: 173012
Antibiotic-Antimycotic Mixed Stock Solution	Nacalai Tesque Inc.	Cat#: 09366-44
Fibroblast Growth Medium 2 Kit	PromoCell	Cat#: C23020
Dulbecco's Phosphate Buffered Saline (D-PBS)	Nacalai Tesque Inc.	Cat#: 14249-24
4%-Paraformaldehyde Phosphate Buffer Solution (PFA)	Nacalai Tesque Inc.	Cat#: 09154-85

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Amido Black 10B powder	Nacalai Tesque Inc.	Cat#: 01927-92
Acetic Acid	Nacalai Tesque Inc.	Cat#: 00212-85
Methanol	Nacalai Tesque Inc.	Cat#: 21915-93
Hydrochloric Acid (HCl, 35%)	Nacalai Tesque Inc.	Cat#: 18320-15
Sodium Hydroxide (NaOH)	Nacalai Tesque Inc.	Cat#: 31511-05
1mol/L Tris-HCl Buffer Solution (pH 8.0)	Nacalai Tesque Inc.	Cat#: 35435-11
Sodium Chloride (NaCl)	Nacalai Tesque Inc.	Cat#: 31319-45
Nonidet® P40 Substitute (NP-40)	Nacalai Tesque Inc.	Cat#: 18558-25
Protease Inhibitor Cocktail (EDTA free) (100x)	Nacalai Tesque Inc.	Cat#: 03969-21
Sodium Orthovanadate (Na ₃ VO ₄)	Nacalai Tesque Inc.	Cat#: 08190-64
Tris(hydroxymethyl)aminomethane (Tris Base)	Nacalai Tesque Inc.	Cat#: 35406-91
Glycine	Nacalai Tesque Inc.	Cat#: 17109-35
Sodium Lauryl Sulfate (SDS)	Nacalai Tesque Inc.	Cat#: 08933-05
Glycerol	Nacalai Tesque Inc.	Cat#: 17018-25
2-Mercaptoethanol	Nacalai Tesque Inc.	Cat#: 21438-82
Bromophenol Blue (BPB)	Nacalai Tesque Inc.	Cat#: 05808-61
Running Buffer Solution(10x) for PAGE	Nacalai Tesque Inc.	Cat#: 30340-91
Albumin, Bovine Serum, pH 5.2 (BSA)	Nacalai Tesque Inc.	Cat#: 01863-48
Polyoxyethylene Sorbitan Monolaurate (Tween® 20)	Nacalai Tesque Inc.	Cat#: 35624-15
BCIP-NBT Solution (Ready To Use)	Nacalai Tesque Inc.	Cat#: 19880-84
Crystal Violet	Nacalai Tesque Inc.	Cat#: 09804-52
Avicel® PH-101	Fluka Analytical (Sigma-Aldrich)	Cat#: 101620601
TRIzol Reagent	Invitrogen	Cat#: 15596018
Lipofectamine® 2000	Thermo-Fisher Scientific	Cat#: 11668500
Lipofectamine® RNAiMAX	Thermo-Fisher Scientific	Cat#: 13778150
Opti-MEM	Thermo-Fisher Scientific	Cat#: 31985070
BbsI	New England Biolabs	Cat#: R0529S
Monarch® DNA Gel Extraction Kit	New England Biolabs	Cat#: T1020
T4PNK	TOYOBO	Cat#: PNK-111
Ligation High	TOYOBO	Cat#: LGK-101
NucleoBond® Xtra Midi	Macherey-Nagel	Cat#: 740410.100
VPg ^{EMCV} (20AA, synthetic peptide)	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{EMCV17AA} (17AA, synthetic peptide)	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{EMCV13AA} (13AA, synthetic peptide)	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{EMCV8AA} (8AA, synthetic peptide)	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{EMCV-F} (20AA, synthetic peptide)	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{P-EMCV} (22AA, synthetic peptide)	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{PV} (22AA, synthetic peptide)	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{TMEV} (20AA, synthetic peptide)	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{Scaffold} (20AA, synthetic peptide)	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{EMCV-G1A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{EMCV-P2A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{EMCV-Y3A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{EMCV-N4A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{EMCV-E5A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{EMCV-T6A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{EMCV-R8A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VPg ^{EMCV-V9A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
VP _g ^{EMCV-K10A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VP _g ^{EMCV-P11A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VP _g ^{EMCV-K12A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VP _g ^{EMCV-T13A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VP _g ^{EMCV-L14A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VP _g ^{EMCV-Q15A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VP _g ^{EMCV-L16A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VP _g ^{EMCV-L17A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VP _g ^{EMCV-D18A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VP _g ^{EMCV-I19A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VP _g ^{EMCV-Q20A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
VP _g ^{EMCV-Q15A/L16A}	This paper (Generated by Eurofins Genomics)	N/A (See Table S2 for sequence)
Z-VAD-FMK (pan-caspase inhibitor)	R&D Systems	Cat#: FMK001
Z-IETD-FMK (caspase-8 inhibitor)	R&D Systems	Cat#: FMK007
Z-LEHD-FMK (caspase-9 inhibitor)	R&D Systems	Cat#: FMK008
Z-DEVD-FMK (caspase-3 inhibitor)	R&D Systems	Cat#: FMK004
Etanercept (ENBREL®) 10mg	Pfizer	Cat#: 45597110
Sephadex® G-25 (Fine)	Sigma-Aldrich	Cat#: G2580
Recombinant Human TNF-α	PeptoTech	Cat#: 300-01A
Recombinant Murine TNF-α	PeptoTech	Cat#: 315-01A
Recombinant Human IFN-β	PeptoTech	Cat#: 300-02BC
Recombinant Human IFN-γ	PeptoTech	Cat#: AF-300-02
L-NAME (hydrochloride)	Cayman Chemical Company	Cat#: 80210
1400W 2HCl	Selleck Chemicals LLC	Cat#: S8337
SIN-1 (chloride)	Cayman Chemical Company	Cat#: 82220
Tofacitinib	AdipoGen Inc.	Cat#: AG-CR1-3625-M005
DNase I, recombinant, RNase-free	Roche	Cat#: 4716728
Phosphodiesterase I from <i>Crotalus adamanteus venom</i>	Sigma-Aldrich	Cat#: P3243
ReverTra Ace® qPCR RT Master Mix with gRNA remover	TOYOBO	Cat#: FSQ-301
THUNDERBIRD SYBR qPCR Mix	TOYOBO	Cat#: QPS-201
Precision Plus Protein Dual Color Standards (Marker)	Bio-Rad	Cat#: 1610374
BLUE Star Prestained Protein-Ladder (Marker)	NIPPON Genetics Co, Ltd.	Cat#: NE-P03
Dynabeads Protein G Immunoprecipitation Kit	Invitrogen	Cat#: DB10007
Tris-Acetate-EDTA Buffer(10x)	Nacalai Tesque Inc.	Cat#: 35430-61
Dimethyl sulfoxide (DMSO)	Nacalai Tesque Inc.	Cat#: 13445-74
Isoflurane	Pfizer	Cat#: 46876352
Critical commercial assays		
Bradford Protein Assay Kit II	Bio-Rad	Cat#: 5000002
Human TNF-alpha ELISA Kit	R&D systems	Cat#: DTA00D
Cell Death Detection ELISA ^{PLUS}	Roche	Cat#: 11774425001
Nitrate/Nitrite Colorimetric Assay Kit	Cayman Chemical Company	Cat#: 780001
CellTiter-Glo® Luminescent Cell Viability Assay	Promega	Cat #: G7572
MycAlert Mycoplasma Detection Kit	Lonza Bioscience	Cat#: LT07-218

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
RNA-seq data	This paper	GEO accession #: GSE286568
Experimental models: Cell lines		
HeLa	ATCC	Cat#: CCL-2; RRID: CVCL_0030
HeLa BAK/BAX DKO	This paper	N/A
HeLa CASP8 KO	This paper	N/A
HeLa IFNGR1 KO	This paper	N/A
HeLa IFNGR2 KO	This paper	N/A
HeLa IFNGR1/2 DKO	This paper	N/A
HeLa TNFR1 KO	This paper	N/A
A549	ATCC	Cat#: CCL-185; RRID: CVCL_0023
RAW 264.7	ATCC	Cat#: TIB-71; RRID: CVCL_0493
L929	ATCC	Cat#: CCL-1; RRID: CVCL_0462
B16-F10	ATCC	Cat#: CRL-6475; RRID: CVCL_0159
Vero	ATCC	Cat#: CCL-81; RRID: CVCL_0059
BHK-21	ATCC	Cat#: CCL-10; RRID: CVCL_1914
HEK293T	ATCC	Cat#: CRL-3216; RRID: CVCL_0063
Experimental models: Organisms/strains		
C57BL/6 mice	CLEA Japan, Inc.	Cat#: C57BL/6JJcl
Oligonucleotides		
See Table S3 for a complete list of oligonucleotides.		N/A
Recombinant DNA		
spCas9(BB)-2A-GFP (PX458)	Ran et al., 2013 ³⁸	Addgene #48138
pCMV6-IFNGR1 (NM_000416) Human Tagged ORF Clone (Myc-DDK-tagged)	OriGene	Cat#: RC202761
pCMV6-IFNGR2 (NM_005534) Human Tagged ORF Clone (Myc-DDK-tagged)	OriGene	Cat#: RC200674
Software and algorithms		
ImageJ	Schneider et al., 2012 ³⁹	https://imagej.net/ij/
GraphPad Prism 10	GraphPad Software Inc.	https://www.graphpad.com
CRISPick	Broad Institute (Doench et al., 2016 ⁴⁰ ; Sanson et al., 2018 ⁴¹)	https://portals.broadinstitute.org/gppx/crispick/public
OlvTools (RNA-seq analysis)	BxINFO	https://olvtools.com
Other		
SH800 Cell Sorter	SONY Biotechnology Inc.	Cat#: SH800
Microplate Reader Model 680	Bio-Rad	Cat#: 168-1000
e-PAGEL Commercial 5–20% SDS-PAGE gel (14 wells)	ATTO Corporation	Cat#: 2331830
e-PAGEL Commercial 5–20% SDS-PAGE gel (18 wells)	ATTO Corporation	Cat#: 2331730
AE-6500 Electrophoresis device	ATTO Corporation	Cat#: 2322190
Trans-Blot SD Semi-Dry Transfer Cell	Bio-Rad	Cat#: 1703940
Immobilon-P (PVDF membrane)	Merck Millipore	Cat#: IPVH08130
Amicon® Ultra Centrifugal Filter, 50kDa MWCO	Merck Millipore	Cat#: UFC9050
Amicon® Ultra Centrifugal Filter, 10kDa MWCO	Merck Millipore	Cat#: UFC8010
Amicon® Ultra Centrifugal Filter, 3kDa MWCO	Merck Millipore	Cat#: UFC8003

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
TriStar ² LB-942 (Multimode microplate reader)	BERTHOLD Technologies	Cat#: LB-942
StepOnePlus TM (Real-Time PCR)	Applied Biosystems	Cat#: StepOnePlus-01
NanoDrop 2000	Thermo Scientific	Cat#: ND-2000

EXPERIMENTAL MODELS AND STUDY PARTICIPANT DETAILS

Cells

HeLa cells (ATCC, CCL-2), A549 cells (ATCC, CCL-185), RAW 264.7 cells (ATCC, TIB-71), L929 cells (ATCC, CCL-1), B16-F10 cells (ATCC, CRL-6475), Vero cells (ATCC, CCL-81), BHK-21 cells (ATCC, CCL-10), and HEK293T cells (ATCC, CRL-3216) were cultured in DMEM medium (Nacalai Tesque Inc., 16971-55) supplied with 10% FBS (Sigma-Aldrich, 173012) and 1% Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque Inc., 09366-44). Human Dermal Fibroblasts, Adult (HDFa, PromoCell, C-12302) were cultured in Fibroblast Basal Medium 2 (PromoCell, C23220) contained in the Fibroblast Growth Medium 2 Kit (PromoCell, C23020), in which the medium was prepared using the manufacturer's instructions. All cell lines were obtained from authenticated commercial sources. Knockout HeLa cell lines generated in this study were derived from authenticated parental HeLa cells (ATCC, CCL-2). Cell lines were regularly tested for mycoplasma contamination using MycoAlert (Lonza, LT07-318) following the manufacturer's instructions, and were confirmed negative for all cell lines used in this study.

Mice

C57BL/6 (ordering name: C57BL/6JJcl) were purchased from CLEA Japan, Inc. All mice were female and 12 weeks old. Mice were maintained under specific pathogen-free (SPF) conditions at all times, and handled in accordance with the regulation on animal experimentation at Kyoto University and the fundamental guideline for proper conduct of animal experiment and related activities in academic research institutions under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science, and Technology. The studies on C57BL/6 mice were reviewed and approved by the Animal Experimentation Committee of Kyoto University (approval number: 253001). Virus infection experiments with mice were conducted in a biosafety level 3 facility.

Viruses

Encephalomyocarditis virus (EMCV) was obtained through ATCC (VR-129B), and amplified in Vero cells (ATCC, CCL-81) in DMEM (Nacalai Tesque Inc., 16971-55) without serum or antibiotics/antimycotics. Virus titer was determined using plaque assay (see Method Details section). Sendai virus (SeV) strain Cantell was obtained through ATCC (VR-907), propagated in chicken embryonated eggs, and virus titer was determined using plaque assay. Newcastle disease virus (NDV) strain Miyadera/51 was identical from the one used in a past study,³³ propagated in chicken embryonated eggs, and virus titer was determined using plaque assay. Vesicular stomatitis virus (VSV) strain Indiana was identical from the one used in a past study,³⁴ and amplified in BHK-21 cells (ATCC, CCL-10) in DMEM (Nacalai Tesque Inc., 16971-55) without serum or antibiotics/antimycotics. Virus titer was determined using plaque assay. Poliovirus (PV, strain type I Mahoney), coxsackie virus B3 (CVB3, strain Nancy), and enterovirus 71 (EV71, strain Isehara VP1-145Q variant) were provided by Satoshi Koike (Tokyo Metropolitan Institute of Medical Science). PV originated from an infectious cDNA clone as described in a past study.³⁵ CVB3 originated from a cDNA clone as described in a past study.³⁶ EV71 VP1-145Q variant (which has higher infectivity toward HeLa) was created from the original wild-type VP1-145E, as described in a past study.³⁷ All experiments using viruses were conducted in a biosafety level 2 or level 3 facility.

METHOD DETAILS

Treatment of cells

Z-VAD-FMK (20 μ M, R&D Systems, FMK001), Z-IETD-FMK (20 μ M, R&D Systems, FMK007), Z-LEHD-FMK (20 μ M, R&D Systems, FMK008), Z-DEVDF-FMK (20 μ M, R&D Systems, FMK004), Tofacitinib (500 nM, AdipoGen Inc., AG-CR1-3625-M005), L-NAME (1 mM, Cayman, 80210), or 1400W (100 μ M, Selleck Chemicals, S8337) were pre-treated 2 h before the experiment. Synthetic peptides (VPgs), recombinant TNF- α (PeproTech, human: 300-01A, murine: 315-01A), and recombinant IFN- γ (PeproTech, AF-300-02) were treated together at indicated concentrations. For treatment of cells with virus-inactivated culture supernatants (see "Viral inactivation methods"), the supernatant was diluted 2-fold (unless indicated otherwise) with DMEM (Nacalai Tesque Inc., 16971-55) supplied with 10% FBS (Sigma-Aldrich, 173012) and 1% Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque Inc., 09366-44), and treated directly to cells for 24 h, unless indicated otherwise. For etanercept treatment, the etanercept-recombinant TNF- α mixture in DMEM, depleted by Protein G beads beforehand was treated directly to cells (see "Neutralization of TNF- α ") for 24h. For treatment with fractions from size-dependent separation (see "Size-dependent separation of compounds"), fractions were treated at a 1:10 concentration (fraction:DMEM with 10% FBS and 1% antibiotic-antimycotics) for 24 h. For treatment with the

antibody-VPg^{EMCV} complexes or VPg^{EMCV}-depleted supernatants: these were diluted 2-fold with DMEM (with 10% FBS and 1% antibiotic-antimycotics) and treated to cells for 24 h.

Transfection

For transfection of PX458 plasmids to HeLa cells: HeLa cells (ATCC, CCL-2) were seeded overnight at a density of 1×10^5 cells/mL in 6-well plates (2 mL/well). 2 μ L of Lipofectamine 2000 (Thermo-Fisher Scientific, #11668500) in 250 μ L of Opti-MEM (Thermo-Fisher Scientific, #31985070), and 1 μ g/mL of plasmid in 250 μ L Opti-MEM were mixed vigorously in a 1.5 mL tube, equilibrated at room temperature for 20 min, then added into the cells with 2 mL DMEM (Nacalai Tesque Inc., 16971-55) supplied with 10% FBS (Sigma-Aldrich, 173012) and 1% Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque Inc., 09366-44). Cells were incubated at 37°C for 24 h before use. For transfection of overexpression plasmids to HEK293T cells: 7.5 μ L of Lipofectamine 2000 (Thermo-Fisher Scientific, #11668500) in 500 μ L of Opti-MEM (Thermo-Fisher Scientific, #31985070), and 6 μ g of plasmid (Myc-IFNGR1 or Myc-IFNGR2) in 500 μ L Opti-MEM were mixed vigorously in a 1.5 mL tube, equilibrated at room temperature for 20 min, then added into the cells with 1.5 mL DMEM (Nacalai Tesque Inc., 16971-55) supplied with 10% FBS (Sigma-Aldrich, 173012) and 1% Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque Inc., 09366-44). Cells were incubated at 37°C for 48 h before use. For transfection of siRNA to HeLa cells: HeLa cells were seeded overnight at a density of 1×10^5 cells/mL in 6-well plates (2 mL/well). 2 μ L of Lipofectamine RNAiMAX (Thermo-Fisher Scientific, #13778150) in 250 μ L of Opti-MEM, and 10 nM of siRNA in 250 μ L Opti-MEM were mixed gently, equilibrated at room temperature for 20 min, then added into the cells with 2 mL DMEM supplied with 10% FBS and 1% Antibiotic-Antimycotic Mixed Stock Solution. Cells were transfected for 48 h at 37°C before use.

CRISPR-Cas9 knockout cell line generation

Backbone plasmid spCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid #48138; <http://n2t.net/addgene:48138>; RRID:Addgene 48138), generated according to this published protocol.³⁸ The plasmid was digested using restriction enzyme BbsI (New England Biolabs, R0529S), then the digested plasmid was extracted using Monarch DNA Gel Extraction Kit (New England Biolabs, T1020). Forward and reverse oligomers were annealed and phosphorylated (using T4PNK, TOYOBO, PNK-111) to target the specific gene (Program: 37°C for 30min for phosphorylation, 95°C for 5min, ramped down 5°C/min to 25°C for annealing). 20-mer targeting sequences (see Table S2 for the complete list) were constructed with the help of CRISPick (Broad Institute). The annealed oligomers were inserted into the cleaved plasmid, and ligation (10 min at room temperature) was performed using Ligation High (TOYOBO, LGK-101). Transformation was performed in DH5 α competent cells (TOYOBO, DNA-903F), and plasmids were sequenced to confirm the insertion of the target sequence into the correct sites in the plasmids. Plasmid DNA was collected and purified using NucleoBond Xtra Midi (Macherey-Nagel, 740410.100), following the manufacturer's protocol. CRISPR-Cas9 plasmids were then transfected into HeLa cells (ATCC, CCL-2) (see "Transfection" for details), and GFP-positive cells were sorted using SH800 (SONY Biotechnology Inc.). Cells were diluted for plating, single-cell colonies were picked, and loss of protein expression/activity was tested by immunoblotting (see "SDS-PAGE and Immunoblotting" for details) for knockout validation.

Amido Black 10B cell survival assay

HeLa cells (ATCC, CCL-2) were seeded at 4×10^5 cells/mL in DMEM (Nacalai Tesque Inc., 16971-55) supplied with 10% FBS (Sigma-Aldrich, 173012) and 1% Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque Inc., 09366-44). After treatment (24 h, unless indicated otherwise), cells were washed with PBS (Nacalai Tesque Inc., 14249-24) twice, fixed with 4% paraformaldehyde (PFA) solution (Nacalai Tesque Inc., 09154-85) for 10 min, and stained for 30 min with Amido Black 10B solution (composed of 0.1% Amido Black 10B powder (Nacalai Tesque Inc., 01927-92), 10% acetic acid (Nacalai Tesque Inc., 00212-85), and 50% methanol (Nacalai Tesque Inc., 21915-93)). After staining, the Amido Black 10B solution was then aspirated completely, excess stains were washed off gently with running water, dried, and plates were scanned to assess survival rates.

Quantitative cell survival analysis

For quantitative cell survival analysis, after Amido Black 10B staining (see "Amido Black 10B cell survival assay" for details), the stains were eluted with 50 mM NaOH, and absorbance values were taken with filters 630 nm–405 nm using a microplate reader (Bio-Rad, 168–1000). Relative cell survival was calculated from the standard curve, in which absorbance was normalized to 100% (control well) and 0% (empty well).

Cell survival analysis by ATP quantification

HeLa cells (ATCC, CCL-2) were seeded at 4×10^5 cells/mL in DMEM (Nacalai Tesque Inc., 16971-55) supplied with 10% FBS (Sigma-Aldrich, 173012) and 1% Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque Inc., 09366-44). After treatment (24 h, unless indicated otherwise), CellTiter-Glo reagent (Promega, G7572) was added at a 1:1 ratio with DMEM, and cells were lysed for 2 min on a shaker. After 10 min of stabilization, luminescence was recorded using TriStar² multimode microplate reader (BERTHOLD, LB-942). Relative cell survival by ATP was calculated from the standard curve, in which luminescence was normalized to 100% (control well) and 0% (empty well).

Bradford assay

Proteins concentrations were quantified using Bradford Protein Assay Kit II (Bio-Rad, 5000002) using BSA as the standard. Absorbance at 595 nm was measured for quantification using Microplate Reader Model 680 (Bio-Rad, 168–1000).

SDS-PAGE and immunoblotting

Samples were lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 8.0 (Nacalai Tesque Inc., 35435-11), 150 mM NaCl (Nacalai Tesque Inc., 31319-45), 10% NP-40 Substitute (Nacalai Tesque Inc., 18558-25)) with a protease/phosphatase inhibitor cocktail (0.1 mM Na_3VO_4 (Nacalai Tesque Inc., 08190-64), 1/100 Protease Inhibitor Cocktail (Nacalai Tesque Inc., 03969-21)). Cell lysates were centrifuged at 16000 x g for 10 min, and the supernatant was collected to remove cell debris. Sample protein concentrations were quantified using Bradford assay (see “Bradford assay”), and diluted to 30 $\mu\text{g}/\text{lane}$, unless specified otherwise. Samples were diluted 2-fold using 2X SDS sample buffer (250 mM Tris-HCl pH 6.8 (Tris-base: Nacalai Tesque Inc., 35406-91), 2% SDS (Nacalai Tesque Inc., 08933-05), 10% glycerol (Nacalai Tesque Inc., 17018-25), 5% 2-mercaptoethanol (Nacalai Tesque Inc., 21438-82), 0.005% bromophenol blue (Nacalai Tesque Inc., 05808-61)), heated for 5 min at 100°C, then separated by electrophoresis in 1X SDS running buffer (Nacalai Tesque Inc., 30340-91) at 200V for 40–70 min at room temperature. SDS-PAGE was performed in commercial 5–20% polyacrylamide gels (ATTO Corporation, 2331830 or 2331730), using an AE-6500 Electrophoresis device (ATTO Corporation, 2322190). After the run, the proteins were transferred using Trans-Blot SD Transfer Cell (Bio-Rad, 1703940) at 100 mA/membrane, 100 min to a PVDF membrane (Merck Millipore, IPVH08130). The PVDF membrane was pre-soaked in methanol (Nacalai Tesque Inc., 21915-93), and transferred using transfer buffer (25mM Tris-base, 192mM glycine (Nacalai Tesque Inc., 17109-35), 20% methanol). After the transfer process, the PVDF membrane was blocked using 5% BSA (Nacalai Tesque Inc., 01863-48) in 1X TBS-T (20mM Tris-HCl pH 8.0, 150mM NaCl, 0.1% Tween 20 (Nacalai Tesque Inc., 35624-15) for 60 min, and incubated with primary antibodies (diluted with 5% BSA in TBS-T) overnight at 4°C. Dilution of primary antibodies: Anti-GAPDH (Santa Cruz Biotechnology, sc-32233): 1:1000, Anti-caspase-8 (CST, #9746): 1:500, Anti-Cleaved PARP (CST, #9541): 1:1000, Anti-VPg^{EMCV} (IBL, This study): 1:5000, Anti-Mengo 3DPol (Santa Cruz Biotechnology, sc-65633): 1:500, Anti-iNOS (Santa Cruz Biotechnology, sc-7271): 1:500, Anti-STAT1 (CST, #9172): 1:1000, Anti-pSTAT1 (Y701) (CST, #9167): 1:1000, Anti-pSTAT2 (Y690) (CST, #4441): 1:1000, Anti-Myc-Tag (CST, #2276): 1:1000, Anti-IkB α (Santa Cruz Biotechnology, sc-371): 1:500, Anti-HSP70 (Santa Cruz Biotechnology, sc-32239): 1:1000, Anti-IFNGR1 (Proteintech, 10808-1-AP): 1:1000, Anti-IFNGR2 (Proteintech, 10266-1-AP): 1:500. The PVDF membrane was washed 3 times for 10 min using TBS-T, then incubated for 1 h at room temperature using AP-conjugated secondary antibodies, either anti-mouse (CST, #7056) or anti-rabbit (CST, #7054) diluted using 5% BSA (1:4000 concentration) in TBS-T. After washing 3 times for 10 min using TBS-T, the membrane was washed quickly using TBS, bands were detected using BCIP-NBT solution (Nacalai Tesque Inc., 19880-84), and the reaction was stopped using purified water. The blots were scanned after drying.

Virus infection

HeLa cells (ATCC, CCL-2) were seeded at indicated concentrations and incubated overnight. Virus infection was performed at indicated MOIs (standard: MOI 0.1, unless specified otherwise) in DMEM (Nacalai Tesque Inc., 16971-55) without serum and antibiotics/antimycotics. For Mock infection, DMEM without serum and antibiotics/antimycotics was treated to cells. After 1 h of adsorption in 37°C, culture medium was removed, and changed to DMEM with 10% FBS (Sigma-Aldrich, 173012) with 1% Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque Inc., 09366-44).

Viral inactivation methods

Acid-inactivation: 24 h post-infection, the culture supernatant was collected, and cells/debris were removed using centrifugation (12,000 g, 5 min). 1N HCl was prepared to acidify the virus-infected media. 1N HCl was added to the culture supernatant to strongly acidify the media to pH 2.0. Media were stored at 4°C for 7 days to inactivate the virus, then neutralized to pH 7.0 by slowly adding 500 mM NaOH. Heat-inactivation: 24 h post-infection, the culture supernatant was collected, and cells/debris were removed using centrifugation (12,000 g, 5 min). The supernatant was then heated at 100°C for 10 min using a heat block, then rapidly cooled on ice. Centrifugal filter inactivation: 24 h post-infection, the virus-infected media were filtered using Amicon Ultra 10K (Merck Millipore, UFC8010) following the recommended manufacturer’s instructions, and flowthrough was collected. For all methods, complete loss of viral activity was confirmed by both observation of the cytopathic effect (CPE) and by plaque assay (see “Plaque assay”).

Plaque assay

Vero cells (ATCC, CCL-81) were seeded with 2×10^5 cells/mL and incubated overnight, unless indicated otherwise. Serial dilutions of the virus-infected supernatant were created (see “Virus Infection” for infection protocol) using serum and antibiotic/antimycotic-free DMEM (Nacalai Tesque Inc., 16971-55). Vero cells were then infected using the diluted culture supernatant. After 1 h of incubation for adsorption, the culture medium was aspirated, then changed into 1.5% Avicel PH-101 (Fluka/Sigma-Aldrich, 101620601) in DMEM with 10% FBS (Sigma-Aldrich, 173012) with 1% Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque Inc., 09366-44). Cells were incubated 24 h at 37°C for VSV and EMCV. 1.5% Avicel was then completely removed, cells were washed with PBS (Nacalai Tesque Inc., 14249-24) and fixed with 4% paraformaldehyde solution (Nacalai Tesque Inc., 09154-85) for 10 min. Wells were washed gently with running water. After drying, 0.5% Crystal Violet (2.5g crystal violet (Nacalai Tesque Inc., 09804-52), 20% methanol

(Nacalai Tesque Inc., 21915-93)) was added to stain the cells for 30 min. The wells were then again washed gently with running water and dried completely. Plaques were observed and counted (triplicates), and PFU/mL were calculated.

Enzyme-linked immunosorbent assay (ELISA)

TNF- α quantification: Culture supernatants or fraction samples were collected for ELISA. Human TNF- α ELISA Kit (R&D systems, DTA00D) was used for quantification of TNF- α , following the manufacturer's instructions. Absorbance at 450 nm was measured for quantification using TriStar² multimode microplate reader (BERTHOLD, LB-942). **Cell death quantification by DNA fragmentation:** HeLa cell lysates were seeded at 1×10^5 cells/mL in 48 well plates. 9 h after treatment with fractions or synthetic VPgs + TNF- α (see "Treatment of cells," "Size-dependent separation of compounds," and "Synthetic peptides"), cells were washed twice using PBS, then lysed using 200 μ L of lysis buffer from the Cell Death Detection ELISA^{PLUS} kit (Roche, 11774425001). Cells were incubated at room temperature for 30 min, centrifuged, and 20 μ L of the supernatants (cytoplasmic fraction) were used for ELISA (Roche, 11774425001), following the manufacturer's instructions. Absorbance at 450 nm was measured for quantification using TriStar² multimode microplate reader (BERTHOLD, LB-942). Cell death levels were estimated by DNA fragmentation, setting the mean values of the negative control values (Mock <50 kDa fraction without TNF- α) – blank values as 1.

Neutralization of TNF- α

10 μ g/mL etanercept (ENBREL, Pfizer, 45597110) was mixed with virus-inactivated culture supernatant (see "Viral inactivation methods"), which was diluted 2-fold using DMEM (Nacalai Tesque Inc., 16971-55) supplied with 10% FBS (Sigma-Aldrich, 173012) and 1% Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque Inc., 09366-44). Neutralization of TNF- α was performed for 2 h, then the etanercept-TNF- α complex was depleted using Dynabeads Protein G (Invitrogen, DB10007), following the manufacturer's instructions from the kit (see "Neutralization and VPg^{EMCV} depletion using pulldown" for details) before treatment to cells.

RNA extraction

RNA was isolated from cells or sera using TRIzol reagent (Invitrogen, 15596018), and followed standard methods of chloroform/phenol extraction-ethanol precipitation for purification of RNA. DNase I (Roche, 4716728) was treated according to the protocol for complete removal of DNA. The extracted and purified RNA was quantified (also tested for RNA degradation) using NanoDrop 2000 (Thermo Scientific, ND-2000).

RT-qPCR

Concentration of the extracted RNA was adjusted after quantification (see "RNA extraction"), and RNA was reverse transcribed using ReverTra Ace qPCR RT Master Mix with gRNA remover (TOYOBO, FSQ-301) following the manufacturer's instructions. Real-time PCR (qPCR) was performed using StepOnePlus (Applied Biosystems). THUNDERBIRD SYBR qPCR Mix (TOYOBO, QPS-201) with specific forward/reverse primers (see Table S3 for the list of primers)^{42–45} were used for quantifying the relative ($\Delta\Delta$ CT, relative to GAPDH) expression levels of RNA.

Size-dependent separation of compounds

HeLa cells (ATCC, CCL-2) were infected with EMCV (MOI 0.1) for 24 h (see "Virus infection"). The inactivated supernatant was filtered first using Amicon Ultra 50K (Merck Millipore, UFC9050) following the recommended manufacturer's instructions. The concentrated fraction (>50 kDa) was discarded, and the flowthrough (<50 kDa) was collected. The flowthrough was then further filtered using Amicon Ultra 10K (Merck Millipore, UFC8010). The concentrated supernatant (<50 kDa > 10 kDa) and the flowthrough (<10 kDa) were both collected. The concentrated supernatant (<50 kDa > 10 kDa) was diluted to its original volume using DMEM (10% FBS, 1% A-A mix). The flowthrough (<10 kDa) was further filtered using Amicon Ultra 3K (Merck Millipore, UFC8003). The concentrated supernatant (<10 kDa > 3 kDa) was diluted to its original volume using DMEM (10% FBS, 1% A-A mix). The collected fractions were treated to HeLa cells at a 1:10 concentration (fraction:DMEM with 10% FBS), with or without 10 ng/mL TNF- α (see "Treatment of cells"). Relative cell survival was calculated by relative ATP levels (see Cell survival analysis by ATP quantification").

Griess quantification

Cell culture supernatants or sera were quantified for total nitrite levels (after reducing all nitrate using nitrate reductase) using Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, 780001), following the manufacturer's instructions. Absorbance at 540 nm was measured using Microplate Reader Model 680 (Bio-Rad, 168–1000) for quantification of nitric oxide, using the nitrite standard provided in the kit.

Synthetic peptides

All synthetic peptides used in this study were generated by Eurofins Genomics. Crude mutant VPgs used in this study for alanine scanning (Figures S3D, S3E and S5E) were generated using the O-PLUS system by Eurofins Genomics. Purity of >95% was confirmed for all other peptides used in this study. All peptide sequences used in this study are shown in Table S2.

Antiviral activity assays

Cell survival: HeLa cells (ATCC, CCL-2) were seeded at 2×10^5 cells/mL and incubated overnight. Next day, serial dilutions (diluting 1/2 each time) of IFN- γ (starting from 10 ng/mL), or VPg^{EMCV}, VPg^{TMEV}, VPg^{Saffold}, and VPg^{PV} (starting from 2 ng/mL) were created, and treated to HeLa cells for 24 h (including non-treated). VSV infection was then performed at MOI 0.1 for 24 h (see “Virus infection” for protocol, including Mock infection). Cells were stained using Amido Black 10B and quantified (see “Amido Black 10B cell survival assay” and “Quantitative cell survival analysis”). Plaque counting: HeLa cells were seeded at 2×10^5 cells/mL and incubated overnight. Next day, VPg^{EMCV}, VPg^{TMEV}, VPg^{Saffold}, VPg^{PV}, VPg^{P-EMCV}, VPg^{EMCV-F}, and VPg^{EMCV8AA} were treated at 1 ng/mL for 24 h (including non-treated). VSV infection was then performed at MOI 0.1 for 24 h (see “Virus infection” for protocol), and the culture supernatant was collected. The collected supernatant was subjected to plaque assay (see “Plaque assay”).

Mice infection model

12 weeks-old female C57BL/6 mice (CLEA Japan) were used for the infection model. Groups were divided into Mock infection + PBS injection, Mock infection + 1400W injection, EMCV infection + PBS injection, and EMCV infection + 1400W injection. 5 mice were used for each group ($n = 5$). On the previous day before infection (−1 dpi), all mice were injected (100 μ L) intraperitoneally (i.p.) with either PBS or 1400W (Selleck Chemicals, S8337) (10 mg/kg, diluted in PBS). The next day (0 dpi), all mice were either mock-infected (PBS) or infected with EMCV (1×10^3 PFU/body, diluted in PBS) by i.p. injection (100 μ L). The next day (1 dpi), mice were again injected i.p. (100 μ L) with either PBS or 1400W. On 2 dpi, blood was collected from the tail from each mouse. Serum was collected by centrifuging the clotted blood, and further used for quantification (see “Griess quantification,” “RNA extraction,” and “RT-qPCR”). On 4 dpi, all mice (that were alive at this point) were euthanized using isoflurane (Pfizer, 46876352), strictly following acceptable euthanasia guidelines. Mice weights were measured, and their conditions were monitored daily.

Confirmation of VPg^{EMCV} by immunoblotting

HeLa cells (ATCC, CCL-2) were seeded at 2×10^5 cells/mL, then infected with EMCV (see “Virus infection” for protocol) at MOI 0.1 for 9 h in 6-well plates. Total culture supernatant (2 mL) was collected, centrifuged to remove debris, and was concentrated to 100X using centrifugal concentration (dry-up) using lysis buffer. The lysates were also collected using 200 μ L lysis buffer (4×10^5 cells in 200 μ L). The 100X concentrated supernatants (1, 2, 4-fold dilution for EMCV, and 1-fold Mock), cell lysates (1, 2, 4-fold dilution for EMCV, and 1-fold Mock), as well as VPg^{EMCV} of known concentrations (serial dilutions starting from 10 ng) as standards were all subjected to immunoblotting (see “SDS-PAGE and immunoblotting”). 20 μ L were applied to each lane.

Phosphodiesterase I (PDEI) digestion of VPg^{EMCV} and immunoblotting

HeLa cells (ATCC, CCL-2) were seeded at 2×10^5 cells/mL, then infected with EMCV (see “Virus infection” for protocol) at MOI 0.1 for 24 h in 6-well plates. Total culture supernatant (2 mL) was collected, centrifuged to remove debris, and filtered using a <50 kDa cutoff filter (Merck Millipore, UFC9050). The filtered EMCV supernatants were then digested using >0.1 U/mL (1/1000 dilution of original stock) snake venom-derived phosphodiesterase I (PDEI, Sigma-Aldrich, P3243) at 37°C for 2 h–24 h, as indicated. Conditions without the addition of PDEI were also prepared. The digested supernatants were then heated at 100°C for 10 min for inactivation of PDEI, then concentrated 100X using a centrifugal evaporator. The concentrated supernatants, as well as VPg^{EMCV} of known concentrations (serial dilutions starting from 10 ng) as standards were all subjected to immunoblotting (see “SDS-PAGE and immunoblotting”). 20 μ L were applied to each lane.

Quantification of VPg^{EMCV} in the EMCV-infected supernatant

PVDF membrane (Merck Millipore, IPVH08130) was pre-soaked in methanol (Nacalai Tesque Inc., 21915-93) for 1 min, soaked in distilled water for 2 min, and equilibrated in 1X TBS-T (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20 (Nacalai Tesque Inc., 35624-15) for 10 min. Filter paper was soaked in 1X TBS-T and the wet PVDF membrane was placed on the filter paper. Synthetic VPg^{EMCV} was adjusted to 1 μ g/mL (1 ng/ μ L) and serially diluted (2-fold), then 1 μ L of known concentrations of VPg^{EMCV} was blotted in triplicates ($n = 3$). The <3 kDa filtrate (EMCV-infected supernatant) from Figure 2A was then concentrated 10X using a centrifugal concentrator, then 1 μ L of the supernatant was blotted ($n = 4$). The PVDF membrane was dried for 2 h, blocked with 5% BSA in TBS-T, incubated with anti-VPg^{EMCV}, and blots were detected using BCIP-NBT (see “SDS-PAGE and immunoblotting” for blocking and antibody incubation protocol). Post-immunoblotting, the dot blot images were opened by ImageJ. The blots were made gray-scale and color-inverted before measurement. Values for integrated density (IntDen) was used for measurement of the blot intensities. A reference curve was created using known concentrations of synthetic VPg^{EMCV} ($n = 3$), and the concentration (ng/mL) of VPg^{EMCV} in the EMCV-infected filtered (<3 kDa) supernatant was quantified from the reference curve ($n = 4$). The total average VPg^{EMCV} concentration in the supernatant was then calculated from the 4 values obtained.

Concentration adjustment of natural and synthetic VPg^{EMCV}

After obtaining the average VPg^{EMCV} concentration in the supernatant (~ 17 ng/mL, see “Quantification of VPg^{EMCV} in the EMCV-infected supernatant”), the concentration was adjusted to be approximately 1 ng/mL using DMEM (10% FBS, 1% A/A mix). This supernatant was then serially diluted 2-fold. Similarly, 1 ng/mL synthetic VPg^{EMCV} solution (in DMEM with 10% FBS and 1% A/A mix) was serially diluted 2-fold. These supernatants (both natural and synthetic VPg^{EMCV}) were then treated (24 h) to HeLa cells directly (to

equal its original concentration). Recombinant TNF- α (10 ng/mL) was also added for each condition. 24 h post-treatment, relative survival for each condition was quantified ($n = 3$) (see “Amido Black 10B cell survival assay” and “Quantitative cell survival analysis”).

Anti-VPg^{EMCV} antibody generation

Polyclonal Anti-EMCV 3B (anti-VPg^{EMCV}) antibody was created by Immuno-Biological Laboratories (IBL), at a final concentration of 1 mg/mL confirmed with low endotoxin levels (<0.3 EU/mg/mL). Peptide with an addition of cysteine in the N-terminal region (C + GPYNETARVKPKTLQLLDIQ) was created, and bovine thyroglobulin was used as the carrier protein. Antibody was pooled from the serum of 3 rabbits, and column-purified (all steps conducted by IBL).

VPg^{EMCV} depletion using pulldown

HeLa cells (ATCC, CCL-2) were either mock-infected or EMCV-infected (see “Virus infection”) at MOI 0.1 for 24 h. The culture supernatants were then treated with heat for virus inactivation (see “Viral inactivation methods”). Dynabeads Protein G from the kit (Invitrogen, DB10007) was resuspended and moved to a tube (50 μ L beads was used for 1 mL of supernatant or solution), and the supernatant was removed. 10 μ g of anti-VPg^{EMCV} antibody was used for each condition, and antibody binding performed following the manufacturer’s protocol. Conditions with the beads, but without the addition of the antibody were also created. The supernatant (binding/washing buffer) was then removed on a magnetic rack. The mock-inactivated and EMCV-inactivated culture supernatants, as well as VPg^{EMCV} solution (2 ng/mL) in DMEM (10% FBS, 1% A-A Mix) were mixed with the bead-antibody complex and were incubated in a rotator overnight. The supernatants and VPg^{EMCV} solution were collected carefully on a magnetic rack not to include the magnetic beads, and these were diluted 2-fold using DMEM (10% FBS, 1% A-A Mix). The diluted supernatants and VPg^{EMCV} solutions were treated to cells for survival assay (see “Treatment of cells,” “Amido Black 10B cell survival assay,” and “Quantitative cell survival analysis”).

Co-immunoprecipitation (binding assay) of IFNGR1/2 and VPg^{EMCV}

HEK293T cells (ATCC, CRL-3216) were seeded overnight at a density of 2×10^5 cells/mL in 6-well plates (2 mL/well), then transfected with Myc-IFNGR1, Myc-IFNGR2, or their combination (see “Transfection”). For [Figure S6A](#): Cells were then treated with either 10X concentrated (using a centrifugal evaporator) < 50 kDa-filtered EMCV supernatant or 100 ng/mL VPg^{EMCV} for 1 h (non-treated conditions also prepared). For [Figure S6B](#): Cells were treated with 100 ng/mL VPg^{EMCV} or VPg^{EMCV-Q15A}. The cells were washed carefully using PBS twice, then samples were lysed in cold 200 μ L NP-40 lysis buffer (50 mM Tris-HCl pH 8.0 (Nacalai Tesque Inc., 35435-11), 150 mM NaCl (Nacalai Tesque Inc., 31319-45), 10% NP-40 Substitute (Nacalai Tesque Inc., 18558-25)) with a protease/phosphatase inhibitor cocktail (0.1 mM Na₃VO₄ (Nacalai Tesque Inc., 08190-64), 1/100 Protease Inhibitor Cocktail (Nacalai Tesque Inc., 03969-21)). The lysates were centrifuged at 16000 $\times$ g for 10 min to remove cell debris. Dynabeads Protein G from the kit (Invitrogen, DB10007) was resuspended and moved to a tube (50 μ L beads was used for 100 μ L lysis buffer). 2.5 μ L of anti-Myc-Tag (CST, #2276) antibody was used for each condition, and antibody binding performed following the manufacturer’s protocol. 100 μ L of the lysis buffer were mixed with the bead-antibody complex and were incubated in a rotator at 4°C overnight. The beads were washed following the manufacturer’s protocol, and eluted using 40 μ L of 1:1 elution buffer (from the kit) and 2X SDS sample buffer (250 mM Tris-HCl pH 6.8 (Tris-base: Nacalai Tesque Inc., 35406-91), 2% SDS (Nacalai Tesque Inc., 08933-05), 10% glycerol (Nacalai Tesque Inc., 17018-25), 5% 2-mercaptoethanol (Nacalai Tesque Inc., 21438-82), 0.005% bromophenol blue (Nacalai Tesque Inc., 05808-61)). The bead-elution buffer mixture was heated for 5 min at 100°C, and the beads were removed using a magnetic rack. 20 μ L of the eluted sample from immunoprecipitation, 10 μ L of the lysate input (1/5 concentration), and 1/5 of the total VPg input were separated by electrophoresis (see “SDS-PAGE and immunoblotting”).

RNA-seq and bioinformatic analyses

For preparation of samples, HeLa cells (ATCC, CCL-2) were seeded at 1×10^5 cells/mL overnight, and were treated with 1000 U/mL recombinant human IFN- β (PeproTech, 300-02BC), 10 ng/mL recombinant human IFN- γ (PeproTech, AF-300-02), or 1 ng/mL VPg^{EMCV} for 24 h (including non-treated as N.T.). Samples were taken in biological duplicates ($n = 2$). RNA was extracted from cells (see “RNA extraction”) and RNA quality check/RNA-seq analysis steps were all conducted by Takara Bio. TapeStation system (Agilent) was used for quality check of RNA, and the library was created using SMART-seq(R) v4 Ultra Low Input RNA Kit (Takara Bio), conducted by Takara Bio (following their own kit protocol). Sequence analysis was performed by using the NovaSeq system (Illumina) following the manufacturer’s instructions for preparation of reagents, and the raw data were converted into FASTQ files (conducted by Takara Bio). DRAGEN Bio-IT Platform (Illumina) was used to generate gene expression data (conducted by Takara Bio). Gene expression data obtained were used for heatmap generation of [Figures S7A](#) and [S8A](#), and for the quantitative data in [Figures S7B](#), [S7C](#) and [S8B](#). Using the FASTQ files, bioinformatics analyses were further conducted using OlvTools (BxINFO, Tokyo, Japan). Transcript abundances were estimated using Salmon against human transcriptome GRCh39. Gene abundances were then calculated from transcript abundances using tximport package. Differential expression analysis was performed using edgeR package after filterByExpr function was applied to remove low expressed genes. The genes with |FC| > 1 and FDR < 0.05 were considered differentially expressed. The volcano plot of [Figure 5A](#) was generated using OlvTools, and data obtained from OlvTools were used for

comparison of top upregulated genes by FDR ranking (Figures 5B and 5C), and for obtaining the top upregulated or downregulated genes for Figures S7A and S8A. RNA-seq analysis data are deposited to GEO (see “Data and Code Availability” & “Key Resources Table”).

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism 10 was used for data analysis. Data points in this study are biological replicates, and data are shown as the mean (SD). For comparison between two groups, statistical significance was determined by two-tailed Student's *t* test. For comparison between three or more groups, statistical significance was determined by one-way ANOVA (for one independent variable) or two-way ANOVA (for two independent variables), followed by Tukey's HSD post hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.